SPERM COMPETITION AND MALE SOCIAL DOMINANCE IN THE BANK VOLE (*MYODES GLAREOLUS*)

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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Declaration of work conducted

All the work presented in this thesis is my own except for **Chapter 3**; some of the preparation of the baculum was performed under my supervision by Miss Nicola Jennings (BVSc student, University of Liverpool) and the preparation of the penile spines and scanning electron microscopy were performed by Miss Meg Stark (Technology Facility, Biology department, University of York).

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Abstract

Sperm competition is a widespread phenomenon where sperm from two or more males compete to fertilize a given set of ova. There is now evidence across diverse taxa that sperm competition is an important selective pressure that has shaped different aspects of male phenotype such as reproductive anatomy, reproductive physiology and copulatory behaviour. In this thesis, I investigate adaptations to sperm competition in a promiscuous rodent, the bank vole (*Myodes glareolus* formerly *Clethrionomys glareolus*). One of the characteristics of bank voles is that males establish dominant and subordinate relationships. Females generally promote multiple mating, such that situations of sperm competition involving dominant and subordinate males are common. However, in a simultaneous choice situation, dominant males are preferred by females. Dominant males therefore have favoured access to females in pre-copulatory competition and theoretical studies predict that male reproductive strategies should vary with their dominance status. I review this general background to the thesis in **Chapter 1**.

I look for evidence of male mate choice in the bank vole in the context of inbreeding avoidance in **Chapter 2**. Because of their low search costs and high encounter rate with sexually receptive females, dominant males are predicted to be less tolerant of inbreeding compared to subordinates. However, males should generally be more stringent in their mate choice when the cost of reproduction increases (e.g. when they are sperm depleted). I report that male bank voles show behaviour that is likely to promote inbreeding avoidance by spending more time in proximity to unrelated females than to siblings, although there is no significant difference in the relative time that dominant and subordinate males spend visiting unrelated and sibling females. Moreover, contrary to theoretical predictions males appear more tolerant to inbreeding when they are in a sperm-depleted state.

Next, I investigate in **Chapter 3**, the role of post-copulatory sexual selection in the evolution of male genitalia. Male bank voles have complex genitalia including a baculum (os penis) and penile spines at the base of the penis. Differences in these structures between dominant and subordinate males could explain the higher reproductive success of dominant males found in previous studies. I found that the basal width of the baculum (but not the length) is significantly larger for dominant male bank voles than subordinates. This trait also exhibits a pattern of positive allometry and a high phenotypic variance, consistent with a role in post-copulatory sexual selection. In contrast, no difference was found in penile spines of dominant and subordinate males, and no evidence was found for positive allometry in this trait.

In **Chapter 4**, I test the theoretical prediction that males mating in a disfavoured role should allocate more sperm in a reproductive event than those mating in a favoured role. However, contrary to these predictions, my comparisons of ejaculate expenditure in relation to male social status demonstrate that dominant male bank voles invest more sperm per ejaculate than

subordinates. Adaptations in reproductive anatomy according to social status are also revealed since dominant males have heavier preputial glands, testes, epididymides, and seminal vesicles (all organ masses controlled for body mass) than subordinate males. However sperm motility does not differ in relation to male social status. Analysis of mating sequences shows that dominant males perform more intromissions prior to ejaculation than subordinates. Dominant males may thus improve their fitness both by investing more sperm per ejaculate and by delivering more stimulation to females.

In **Chapter 5** I investigate how male bank voles respond to average risk of sperm competition by manipulating their social experience through the number of competitors odours encountered in the environment. I find no difference in male investment in sperm production or sperm motility according to average sperm competition risk. Nevertheless, I find that males exposed to 'high' competition have larger seminal vesicles than those exposed to 'low' competition, suggesting that male bank voles might benefit from an elevated quantity of seminal fluid protein in their ejaculate when the average risk of sperm competition in the population is high.

I use a comparative approach to test the hypothesis that sperm competition influences brain size evolution in mammals in **Chapter 6**. No evidence of a trade-off between brain and testis mass is found in rodents, ungulates, primates, carnivores, or across combined mammalian orders. Although previous reports of a negative relationship between brain and testis mass in echolocating bats are confirmed in this study, mating system appears to be a better predictor of brain size in this group.

Overall, results of this thesis imply that the reproductive strategies of male mammals vary with both expected average levels of sperm competition and individual competitive ability. The higher reproductive success of dominant male bank voles may therefore be explained through their greater investment in sperm competition but also perhaps via female cryptic choice. However, seminal vesicle products might also play an important role in post-copulatory sexual selection and now deserve more attention in the study of sperm competition in bank voles and more generally in mammals. Finally, opportunities for further studies are discussed in **Chapter 7**.

Key-Words: Sexual selection; Sperm competition; Bank vole; *Myodes* = *Clethrionomys glareolus*; Mate choice; Testis size; Seminal vesicle; Brain size; Comparative studies; Genitalia.

Chapter 1: General introduction

1.1 Chapter overview

This opening chapter will first introduce the main concepts addressed in this thesis such as sexual selection, sperm competition, female cryptic choice and sexual conflict. I will then present an overview of the literature concerning the evolutionary consequences of sperm competition for different male reproductive traits (i.e. testis size, sperm phenotype, and copulatory behaviour), particularly in mammals. Since the principal subject of the thesis will be a promiscuous rodent in which males form dominance relationships, I will describe the importance of male social dominance in sexual selection and its link with male reproductive success. This section will be limited to an overview of the recent mammalian research in this area. Finally I will explain the relevance of my biological model, the bank vole (*Myodes glareolus*) in the context of sexual selection studies and more particularly post-copulatory sexual competition, by reviewing the ecology, social structure, mating system and the occurrence of sperm competition in this species. A brief overview of the different chapters of the thesis will close this introductory section.

1.2 Sexual selection

(a) Sexual selection

Darwin (1859) introduced the concept of sexual selection[1], and presented it in '*The Descent of Man and Selection in Relation to Sex*' (Darwin 1871) to explain the sexual dimorphism observed in a large variety of species such as ungulates: '*When the males are provided with weapons which in the females are absent there can hardly be a doubt that these serve for*

[1] To date, the most recent definition of sexual selection found in a textbook is '*Selection among variants that occurs according to their ability at securing access to the gametes of the other sex*' (Danchin *et al.* 2008) but note that there is currently a debate around the definition of sexual selection (Clutton-Brock 2007; Carranza 2009; see text).

fighting with other males; and that they were acquired through sexual selection, and were transmitted to the male sex alone' (Darwin 1871 pp 564). Because such conspicuous traits should be counterselected by natural selection, Darwin suggested that they should provide benefits in sexual competition. However, it took several decades after Darwin's pioneering thoughts to refine the explanations of the evolution and maintenance of these exaggerated traits through the 'runaway process' (Fisher 1930; Lande 1981) or the 'handicap theory' (Zahavi 1975, 1977). Similarly, if it is nowadays established that males compete to have access to females, theoretical frameworks designed to understand differences between the sexes and how sexual selection acts on each sex differently have been developed only recently (Williams 1966; Trivers 1972; review in Bateson 1983; Andersson 1994) leading to a consensus around 'choosy' females and 'indiscriminate' males (Andersson 1994; Andersson & Iwasa 1996). The first empirical evidence on sex-role differences came from Bateman's (1948) experiments in *Drosophila melanogaster*, who found two fundamental results for the development of sexual selection theory: a greater variance in reproductive success for males than for females and that male fertility is correlated with the number of matings he obtains which is not the case for female fertility (Bateman 1948; but see Dewsbury 2005).

(b) Sexual selection and sex-roles

Females and males invest differently in reproduction. The traditional view of anisogamy is that females invest a relatively large quantity of energy in only a few gametes (i.e. eggs) whereas males produce a much larger number of relatively cheap gametes (i.e. sperm). Even if nowadays the idea of an extremely low investment in reproduction by males tends to be moderated (Dewsbury 2005; Tang-Martinez & Ryder 2005), males typically invest less than females in parental care (Trivers 1972; Clutton-Brock 1991). Therefore, the operational sex-ratio (OSR) defined by Emlen and Oring (1977) as the average ratio of fertilizable females to sexually active males at any given time in a population is generally biased toward males (Emlen & Oring 1977), and males are in competition to have access to the rare resource constituted by females (Emlen & Oring 1977; Andersson 1994). Notwithstanding, since parental investment and the operational sex-ratio are difficult to estimate in natural populations, Clutton-Brock and Vincent (1991) advocated to use the potential reproductive rate (PRR), measured as the maximum number of independent offspring that parents can produce per unit time (Clutton-Brock & Vincent 1991). Operational sex-ratio and potential reproductive rate are two related measures (Clutton-Brock & Parker 1992; **Figure 1.1**; but see

Kokko & Monaghan 2001), both mediated by the availability of each sex for mating opportunities during its reproductive cycle (i.e. 'Time in' period, versus the 'Time out' period when individuals are not available for mating opportunities) (Clutton-Brock & Parker 1992). Since in a large range of taxa, the period of receptivity of the female is relatively short compared to males (e.g. Gomendio *et al.* 1998), males have a higher potential rate of reproduction and therefore are in competition for females, who are in turn predicted to choose high quality males (Clutton-Brock & Parker 1992; Andersson 1994). The potential rate of reproduction is a powerful tool to predict the direction of sexual selection. For example, in some species such as pipefish (commonly named 'sex-role reversed' species), males carry the eggs and females have a higher potential reproductive rate because they can produce eggs at a faster rate than males can carry eggs (e.g. Berglund *et al.* 1986; Berglund *et al.* 1989). Therefore, in such rare species, females are in competition for access to males (Clutton-Brock & Vincent 1991; Vincent *et al.* 1992). However, even if the OSR and the PRR are two reliable tools to predict the direction and intensity of sexual selection in many studies (e.g. Gwynne & Simmons 1990; Clutton-Brock & Vincent 1991), other factors can influence the direction of sexual selection (Clutton-Brock 2007), such as differences in mortality rates between sexes (Clutton-Brock & Parker 1992) and the variance in male and female quality (Johnstone *et al.* 1996; Tang-Martinez & Ryder 2005). Recently, Kokko and Monaghan (2001) suggested that the sex-specific cost of breeding might be the best predictor of the direction of sexual selection, even if OSR and PRR are generally valid predictors since they covary strongly with the cost of breeding (Kokko & Monaghan 2001).

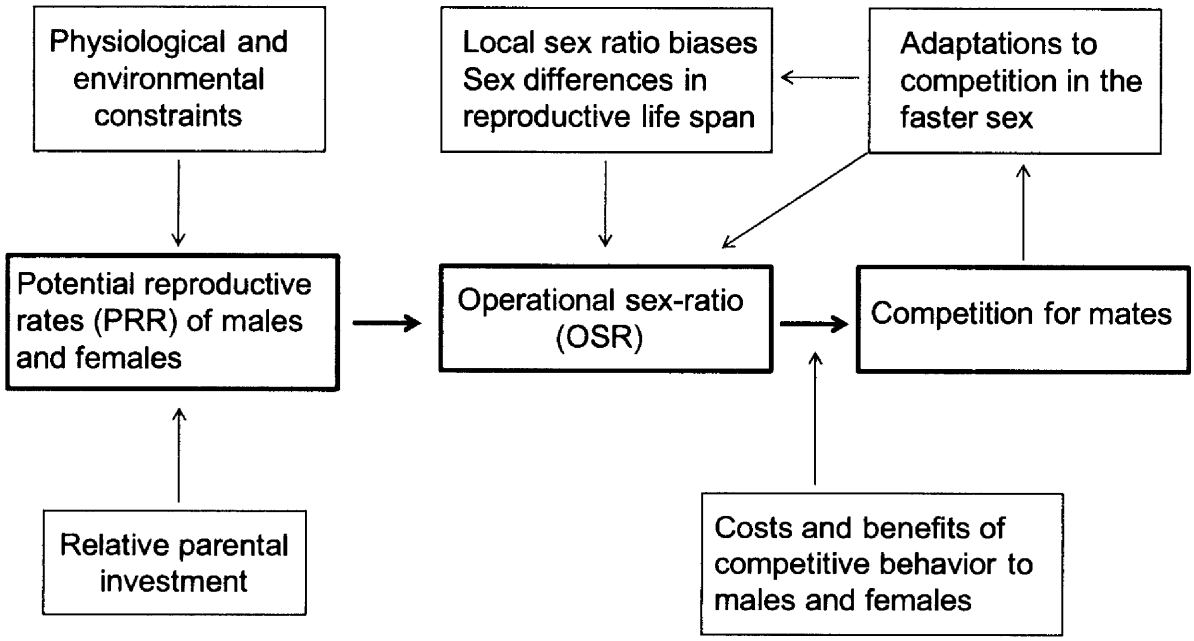

Figure 1.1 Diagram of the relationship between mating competition, operational sex-ratio and potential rates of reproduction by males and females, and relative parental expenditure by the two sexes (modified from Clutton-Brock & Parker 1992).

(c) Pre-copulatory sexual selection

Pre-copulatory competition can take different forms such as competition between individuals of the same sex for mating opportunities (intrasexual selection) or to attract mates (intersexual selection) (Andersson 1994). As pointed out above, it is generally males who will compete to have access to females, which explains why ornaments and armaments are more likely to be found in this gender (Darwin 1871; Andersson 1994). In mammals, intrasexual selection is predominant (Clutton-Brock & McAuliffe 2009) and many traits have evolved in males to indicate their body condition and fighting abilities to opponents, such as antler size in ungulates (Clutton-Brock 1982) or more specifically and surprisingly knee-click frequency in the eland (*Tragelaphus oryx*) (Bro-Jørgensen & Dabelsteen 2008). However, in mammalian species, as for other groups, female choice can occur for a large variety of traits such as dominance, symmetry, relatedness, genetic compatibilities and vocal or olfactory displays (review in Clutton-Brock & McAuliffe 2009). To illustrate this, females red deer (*Cervus elaphus*) are more attracted by the roar of large males (Charlton *et al.* 2007) and female house mice (*Mus musculus*) show a preference for MUP (Major Urinary Protein) heterozygous males (Thom *et al.* 2008) based on chemical signals conveyed through urine (Thom *et al.* 2008; Hurst 2009). Until recently, the study of female preferences has been the principal

focus of sexual selection studies and the study of male mate choice has been mainly limited to the case of sex-role reversed species (Clutton-Brock & Vincent 1991). However, recent reviews have pointed out that male choice and female competition can be more widespread than commonly assumed (Clutton-Brock 2007; Clutton-Brock 2009). This bias in mate choice studies might be attributed to the influence of Darwin (1871) and Bateman (1948), who emphasized the importance of mate choice by females in sexual selection (Dewsbury 2005; Clutton-Brock 2009). Empirical and theoretical studies have revealed that male (or mutual) mate choice is expected to evolve under certain conditions, if reproduction is costly for males (Dewsbury 1982a; Kokko & Johnstone 2002), if males have a high probability of attaining further reproductive opportunities (Owens & Thompson 1994; Chenoweth *et al.* 2006) or if there is large variation in female quality (Parker 1983; Johnstone *et al.* 1996). There is now evidence that male mate choice does indeed occur in a large range of species (Clutton-Brock 2009) from invertebrates (see Bonduriansky 2001 for a review in insects) to vertebrates (e.g. Hill 1993 in birds; Amundsen & Forsgren 2001 in fishes; Muller *et al.* 2006 in mammals). For example, in the Pacific blue-eye fish (*Pseudomugil signifer*), males are choosy and show a preference for larger females since they are more fecund (Wong & Jennions 2003). Interestingly, this preference increases with search costs (Wong & Jennions 2003). Such variations in male mate choice according to various costs associated with mating are not uncommon (e.g. Byrne & Rice 2006) and will be explored further in **Chapter 2**.

(d) Female multiple mating and sperm competition

In '*The Descent of Man and Selection in Relation to Sex*' (Darwin 1871), the view of male competition for access to females is limited to pre-copulatory sexual competition[1]. However, female multiple mating is now known to be widespread in the animal kingdom since females often benefit from polyandry (review in Hosken & Stockley 2003). There is sometimes confusion in the use of the term 'polyandry' and in this thesis it will refer to female propensity to mate multiply (Birkhead & Møller 1998; Hosken & Stockley 2003) not to the mating system *stricto sensu* (review in Shuster & Wade 2003; Cézilly & Danchin 2008). Even if male coercion is sometimes possible (Hosken & Stockley 2003), females generally initiate multiple mating since it can for example improve their lifespan (Brown 1997; Hosken & Stockley

[1] It might be possible that Charles Darwin was aware of the female propensity to mate multiply but that the moral constraints of the Victorian society had prevented him to mention such examples in his book (Birkhead 2000).

2003), their offspring number (Osikowski & Rafiński 2001; Byrne & Whiting 2008) or quality (Jennions & Petrie 2000; Cornell & Tregenza 2007) and decrease the risk of infanticide (Hosken & Stockley 2003; Klemme & Ylönen 2010)[1]. Therefore, ejaculates from different males are likely to be present at the same time in the female reproductive tract and sperm from these ejaculates will be in competition to fertilize the ova. This sperm competition was first pointed out by Parker (1970a) and is commonly defined as "*The competition between the sperm from two or more males for the fertilization of a given set of ova*" (Parker 1998). Sperm competition is a pervasive evolutionary force that has shaped several aspects of male phenotype (review in Birkhead & Møller 1998; Simmons 2001; **Section 1.3**). Although the first theoretical predictions appeared in the early 70s (e.g. Parker 1970a,b), empirical work focused on this topic only emerged in the 80s, mainly in insects (Smith 1984a; Simmons 2001). Sperm competition is generally compared to a raffle where the probability to win the competition is proportional to the number of sperm invested in an ejaculate (Parker 1998). However as I will discuss in **Section 1.3**, sperm number is not the only determinant of male fertilization success under sperm competition (Snook 2005).

(e) Sperm competition, cryptic female choice and sexual conflict

Similar to what happens before copulation, intrasexual selection can also occur in the female reproductive tract, via cryptic female choice (a concept first coined by Thornill 1983) where females can be selective toward sperm from specific males and bias paternity toward these males (review in Eberhard 1996). This choice can take place between insemination and fertilization or after fertilization (Wedekind 1994). In the fowl (*Gallus gallus*), Pizzari and Birkhead (2000) found that all else being equal, females eject more sperm from subordinate males than from dominant males. They argued that this is an adaptive strategy because females gain benefits from having their eggs fertilized by dominant males (Pizzari & Birkhead 2000). Such paternity bias toward better quality males is also under female control in scorpionflies (*Panorpa vulgaris*) (Vermeulen *et al.* 2008). Because sperm competition and cryptic choice can take place at the same time, it is always difficult to tease apart the effect of these two processes on the relative siring success of each male (Birkhead 1998; Eberhard 1998). Moreover, the interaction between sperm competition and female cryptic choice might be dynamic, for example, it is likely that strategic sperm allocation (see also **Section 1.3.d**)

[1] The list of the benefits of polyandry is not thorough here and more detailed explanation can be found in Smith (1984b), Jennions and Petrie (2000) and Hosken and Stockley (2003).

has evolved in species where females exhibit a cryptic choice in order to counteract the effects of this choice (e.g. Holman & Snook 2006). These examples show that males and females diverge in their evolutionary interests and lead to the question of intersexual conflict (Parker 1979; Arnqvist & Rowe 2005). Indeed, there is now evidence that sexual conflict is a selective force that drives the evolution of different reproductive tactics in males and females (East *et al.* 2003; Arnqvist & Rowe 2005). For instance, in several species, males and females differ in their optimal number of copulations and females can suffer fitness costs of an increased number of copulations due to increased risk of sexually transmitted diseases or other costs (review in Arnqvist & Rowe 2005). This difference in the optimal number of copulations between the sexes has promoted the evolution of adaptations to persistence in males and resistance in females (Inceoglu *et al.* 2003; Arnqvist & Rowe 2005).

Interestingly, sexual conflicts often arise from adaptations to sperm competition (Stockley 1997; Arnqvist & Rowe 2005). For example, in the dipteran *Drosophila montana*, males benefit from prolonged copulations when they face a risk of sperm competition since it will delay female remating (Mazzi *et al.* 2009). However, females try to reduce the duration of the copulation by dislodging their partner through leg kicking and wing flicking (Mazzi *et al.* 2009).

1.3 Sperm competition: theory and empiry

(a) Evolutionary consequences of sperm competition

Sperm competition occurs in a wide variety of taxa (review in Smith 1984a; Birkhead & Møller 1998; Simmons 2001; Shackelford & Pound 2005) and is a powerful evolutionary force that has shaped different aspects of male phenotype such as reproductive anatomy, physiology and behaviour (Birkhead & Møller 1998). It is also acknowledged now that the relationship between mating systems and sperm competition is dynamic (Cézilly & Danchin 2008). Indeed, as underlined above in promiscuous and polyandrous species, female multiple mating promotes sperm competition (Birkhead & Møller 1998; Shuster & Wade 2003). Besides, sperm competition can also affect the mating system. In birds, for example, the last male to mate often has an advantage in sperm competition (Gomendio *et al.* 1993). Therefore mate guarding, initially performed to prevent female remating, is likely to have evolved into stable social bonds (Gomendio *et al.* 1993). A monogamous mating system is also the

consequence of prolonged mate guarding in the dik-dik (*Madoqua kirkii*) (Brotherton & Manser 1997), but this relationship is less frequent in mammals than in birds (Gomendio *et al.* 1993; Cézilly & Danchin 2008).

Sperm competition favours adaptations to prevent female remating and/or adaptations to improve sperm competitiveness (Birkhead & Møller 1998; Simmons 2001; Pizzari & Parker 2009; Pitnick *et al.* 2009). For example, in insects and spiders, genital adaptations to sperm competition are widespread (review in Eberhard 1985; Aisenberg & Eberhard 2009; Řezáč 2009) and will be described in more detail in **Chapter 3**. Interestingly, sperm competition also plays a role in the evolution of sperm phenotype (Pizzari & Parker 2009). Indeed, the proportion of motile sperm and variation in sperm swimming velocity in an ejaculate are important in the fertilization success of an individual (review in Snook 2005; Pizzari & Parker 2009). For example, in fishes such as the Atlantic salmon (*Salmo salar*), higher sperm velocity confers an advantage in sperm competition (Gage *et al.* 2004; see also Fitzpatrick *et al.* 2009). In this group, sperm velocity can also be a plastic trait, adjusted in relation to the number of mating opportunities (Gasparini *et al.* 2009). Nevertheless an increase in sperm motility might be costly in terms of sperm longevity (review in Pizzari & Parker 2009).

Moreover, sperm size, another important determinant of the outcome of sperm competition (e.g. Garcia-González & Simmons 2007; Tourmente *et al.* 2009) may also influence sperm motility (Snook 2005; Pizzari & Parker 2009, see **Section 1.3.b**). Although comparative studies show that sperm motility is positively correlated with sperm length in birds (Lüpold *et al.* 2009), this pattern is not found in fishes (Gage *et al.* 2002) and there is no consensus yet on the relationship between these two traits (Pizzari & Parker 2009). However, Fitzpatrick *et al.* (2009) have recently suggested a scenario with increases in sperm motility evolving first and sperm size next to sustain the energy demand required for a rapid swim (see also Pizzari 2009). Confirming this evolutionary relationship between sperm motility and size, an intra-specific study in the zebra finch (*Taeniopygia guttata*) found that both traits were heritable and genetically correlated (Mossman *et al.* 2009).

(b) Sperm competition in mammals

Sperm competition is widespread in mammals (review in Gomendio *et al.* 1998; Taggart *et al.* 1998). Because it is difficult to infer the likelihood of sperm competition on the basis of social

organization in this group (Gomendio *et al.* 1998; but see Cohas & Allainé 2009), evidence that females mate with different males in the same oestrus period is generally found through the use of molecular tools such as DNA fingerprinting (e.g. cheetahs, *Acinonyx jubatus*: Gottelli *et al.* 2007; treeshrews, *Tupaia tana*: Munshi-South 2007; raccoons, *Procyon lotor*: Nielsen & Nielsen 2007). One of the typical traits of mammals is that females are receptive for only a short period of time (Gomendio *et al.* 1998). Therefore, to take part in the competition, males have to copulate with the female in this short period of time (Gomendio *et al.* 1998). Moreover, sperm life-span is generally short (Austin 1975; Parker 1984) and needs to match the period of female receptivity (Bishop 1969; Gomendio & Roldan 1993; Gomendio *et al.* 1998), except for bats since in this group sperm may be stored for months in the female tract (Fenton 1984). Males will typically maximize their chances of fertilization by mating at an optimal time, i.e. close to ovulation but allowing time for capacitation to occur (Chen *et al.* 1989; Gomendio *et al.* 1998), although the mating position (generally first or last) can in some species also be important (Schwagmeyer & Foltz 1990; Lacey *et al.* 1997).

In mammals, sperm competition has evolutionary consequences mainly on three different aspects of male phenotype: structure of the genitalia, reproductive physiology and sexual behaviour (Dixson & Anderson 2004; Stockley 2004). In the next two sections, I will describe the evolutionary consequences of sperm competition on relative testis size, sperm morphology/motility and copulatory behaviour in mammals. The influence of sperm competition on the evolution of mammalian genitalia will be presented in more detail in the introduction of **Chapter 3**.

i) Testis size, sperm morphology and motility

To consider again Parker's comparison between sperm competition and a raffle (Parker 1998), the more 'tickets' (i.e. sperm) a male invests into the copulation, the greater his chances of winning the competition (Parker 1984; Parker 1998). Therefore, males from species who typically face a high level of sperm competition are predicted to invest more sperm in their ejaculates (Parker 1998), which will require bigger testes to produce them (Møller 1988a). This prediction is supported by several comparative studies across different mammalian taxa where a positive relationship between relative testis mass (controlled for body mass) and the level of sperm competition has been found (see Harcourt *et al.* 1981, Harcourt *et al.* 1995 for primates; Ginsberg & Rubenstein 1990 for ungulates; Ramm *et al.* 2005 for rodents; Hosken

1997, Hosken 1998 for bats; Iossa *et al.* 2008 for carnivores; Rose *et al.* 1997 for marsupials and monotremes; Parapanov *et al.* 2009 for insectivores). These studies use different proxies to assess the level of sperm competition such as mating system (e.g. Ginsberg & Rubenstein 1990), multiple paternity prevalence (e.g. Ramm *et al.* 2005) or length of the mating season (e.g. Iossa *et al.* 2008).

Because sperm number is not the only determinant of the outcome of fertilization success (Snook 2005; Gomendio *et al.* 2006), sperm competition has also shaped different aspects of mammalian morphology and physiology (Stockley 2004; Dixson & Anderson 2004). Currently, one important subject of debate is the evolution of sperm phenotype through sperm competition (review in Pizzari & Parker 2009). Indeed, it is often suggested that longer sperm should be favoured under sperm competition since longer sperm are supposed to swim faster (Snook 2005; Pizzari & Parker 2009) and therefore are more likely to reach the ova first (Gomendio *et al.* 1998). Although it was initially suggested that mammalian species experiencing a high level of sperm competition had longer sperm (Gomendio & Roldan 1991) and more particularly a longer midpiece, at least in primates (Anderson & Dixson 2002), a comparative study across 83 mammalian species failed to find a relationship between sperm size (or the size of any sperm component) and the level of sperm competition (measured as relative testis size) (Gage & Freckleton 2003). The midpiece has been the centre of interest of some studies since it is the part of the sperm that contains the mitochondria that provide the adenosine triphosphate (ATP) essential for flagellum undulation (review in Cummins 2009); however contrasting results between studies (Anderson & Dixson 2002, Gage & Freckleton 2003; Anderson *et al.* 2005) mean that the relationship between sperm morphometry, sperm motility and sperm competition in mammals is still unclear (Stockley 2004). Interestingly, a recent study in closely related species of *Mus* found that the level of sperm competition experienced is strongly associated with the rate of divergence in *protamine 2*, a gene that influences sperm velocity (Martin-Coello *et al.* 2009). Therefore, even if further studies are required to understand the overall relationship between sperm phenotype and sperm competition in mammals, it appears that sperm phenotype has evolved under sexual selection pressures (Parker 1982; Pizzari & Parker 2009). For instance, sperm morphology may be adapted to allow a male's sperm to swim faster (Moore *et al.* 2002).

In mammals, evidence for individual variation in sperm length and motility has only recently been reported. In the Iberian red deer (*Cervus elaphus hispanicus*), sperm with longer

midpieces swim more slowly whereas the length of the head and tail of the spermatozoa are positively correlated with motility (Malo *et al.* 2006), which is an important determinant of reproductive success in this species (Malo *et al.* 2005a). This seems surprising since the midpiece is generally considered to be an important part of the sperm that is under sexual selection (Anderson & Dixson 2002), which is supported by an intra-species study in red squirrels (*Tamiasciurus hudsonicus*) (Bonanno & Schulte-Hostedde 2009). In house mice, males with shorter sperm have an advantage in sperm competition but only when they mate in second position (Firman & Simmons 2008a), and sperm from dominant males swim faster than sperm from subordinates (Koyama & Kamimura 1999; Koyama & Kamimura 2000).

ii) Copulatory behaviour

Sperm competition appears to have also influenced the evolution of copulatory behaviour (Anderson & Dixson 2004; Stockley 2004; Stockley & Preston 2004) and interestingly, in mammals, even closely related species can have highly different patterns of copulatory behaviour (Dewsbury 1975; **Figure 1.2**). In species from the genus *Microtus*, the gray-tailed vole (*Microtus canicaudus*) and the montane vole (*Microtus montanus*) need multiple intromissions to attain an ejaculation whereas the tundra vole (*Microtus oeconomus*) and the taiga vole (*Microtus xanthognathus*) do not (Dewsbury 1973; Dewsbury 1982b). In mammals, copulatory behaviour can be classified by different features such as the presence/absence of a lock, the presence/absence of thrusts and the number of intromissions and ejaculations (Dewsbury 1972; **Figure 1.2**). Because the presence and intensity of these traits vary between species, patterns of copulatory behaviour are extremely diverse in mammals (Dewsbury 1975; Stockley & Preston 2004; **Figure 1.2**) but in most cases, ejaculatory series include several mounts, several intromissions, and an ejaculation at the end (Dewsbury 1975). There is generally no sperm transfer during intromissions (Dewsbury 1972) but the stimulation conferred by these intromissions probably plays an important role in facilitating sperm transport and/or ovulation stimulation (review in Stockley & Preston 2004). For example, a faster pace of copulation may provide an advantage in terms of sperm competition (Toner & Adler 1986). It is also predicted that multiple ejaculations should be found in species where the timing of ovulation is unknown, thereby allowing males to spread sperm delivery across a longer period (Parker 1984) and/or to deliver more sperm (Parker 1984; Ginsberg & Rubenstein 1990).

In a comparative study of rodents, Stockley and Preston (2004) found that the rate, but not the number of intromissions is positively correlated with sperm competition intensity. These authors also found that in species with multiple intromissions, ejaculation latencies were reduced in species with intense sperm competition (Stockley & Preston 2004). Males who face a high level of sperm competition are consequently able to ejaculate more rapidly and to avoid the risk of take-over by a rival male (Stockley & Preston 2004). If sperm competition has influenced copulatory behaviour in rodents, one may also expect to find some intraspecific variation according to the risk and/or intensity of sperm competition (see **Section 1.3.c**). In meadow voles (*Microtus pennsylvanicus*), males adjust their sperm investment according to the risk of sperm competition but no change occurs in terms of total duration of copulation or number of ejaculations (six on average in this species) (delBarco-Trillo & Ferkin 2004) whereas in house mice (*Mus musculus*), males alter their copulatory behaviour (Preston & Stockley 2006). Indeed, in the presence of a rival, male house mice decrease the number of both mounts and intromissions prior to their first ejaculation (Preston & Stockley 2006). On the other hand, these males are more likely to ejaculate twice than males who mate in absence of rivals (Preston & Stockley 2006). Such behaviour may reduce the risk of losing mating opportunities by ejaculating quickly but also may increase the chance to attain paternity by ejaculating repeatedly (Preston & Stockley 2006; but see Ramm & Stockley 2007).

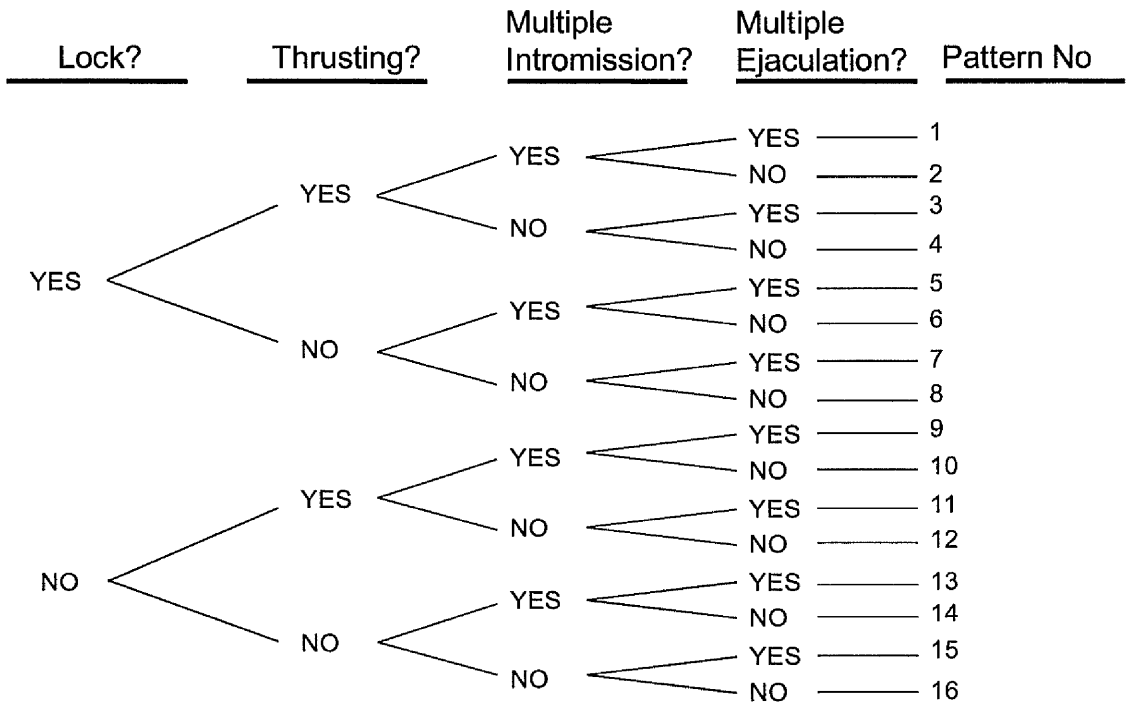

Figure 1.2 Patterns of copulatory behaviour in mammals (modified from Dewsbury 1972). The copulatory behaviour of bank voles matches pattern No 13 on the figure.

(c) Sperm competition games

Since Parker's (1970a) pioneering publication, sperm competition has been the subject of numerous theoretical studies (review in Parker 1998). For instance, models have been elaborated in relation to the 'risk' or 'intensity' of sperm competition (e.g. Parker 1982; Parker *et al.* 1996; Parker *et al.* 1997), the probability of male or female sperm limitation (e.g. Shapiro & Giraldeau 1996; Ball & Parker 2007) and female mating status (Engqvist & Reinhold 2006; Ball & Parker 2007). These models are elaborated in the context of evolutionary game theory using an evolutionarily stable strategy (ESS) approach (Maynard Smith 1982; Parker 1998). They assume that males are limited in their sperm production (Parker 1998) which is confirmed by empirical studies (e.g. Preston *et al.* 2001; Ambriz *et al.* 2002; Lewis & Wedell 2007) and that the quantity of energy allocated for total reproductive expenditure is fixed (Parker 1998). Therefore, males have to trade-off between the two components of reproductive investment: investment in gaining matings and investment in sperm allocation (Parker 1998; **Figure 1.3**). Recent evidence of this trade-off has been discovered in a beetle (*Onthophagus nigriventris*) where males who had their horn

development experimentally altered invested more in testis production (Simmons & Emlen 2006). As emphasized above, male optimal sperm allocation has been studied in different contexts. Here, I will limit descriptions to a general view of the 'risk' and 'intensity' models (Parker 1982; Parker *et al.* 1996; Parker *et al.* 1997) since they have been extensively tested (review in Wedell *et al.* 2002; see also **Section 1.3.d**) and to the 'raffles and roles' models (Parker 1990a) since the experimental work described in **Chapter 4** is directly based on these models.

i) Risk and intensity models

Models of 'risk' and 'intensity' of sperm competition have been tested in a wide range of taxa (Wedell *et al.* 2002). Sperm competition 'risk' (SCR) models apply to species where females mate with a maximum of two males but with a variable probability of double mating variable (Parker *et al.* 1997; Parker 1998). Within this species, this model distinguishes between two 'risks' of sperm competition: 'past' SCR when the female may have already mated and 'future' SCR when the female may mate again. Theory predicts that males should increase their sperm allocation (i.e. sperm number) when the risk (past or future) of sperm competition is increased (Parker *et al.* 1997; Parker 1998). In contrast, sperm competition intensity models apply to species in which sperm competition is extremely likely to occur and where ejaculates of n males (with $n \geq 2$) are in competition to fertilize a set of ova. Therefore, in these models, the risk of sperm competition is always very high (Parker *et al.* 1996; Parker 1998). Counterintuitively, theory predicts that in such a situation, when males have information about competition (limited or perfect information), their sperm expenditure per female should decrease when the estimated number of competitors is above two (Parker *et al.* 1996). Both models, 'risk' and 'intensity', have generally received support in a relatively wide range of taxa (see review in Wedell *et al.* 2002) even if some exceptions have been reported (e.g. Evans 2009). To illustrate this, the cricket (*Teleogryllus oceanicus*) increases sperm expenditure in the presence of one rival whereas the number of live sperm per ejaculate decreases when the intensity of sperm competition increases as predicted by theory (Simmons *et al.* 2007). More recently, it has been suggested that sperm allocation and sperm competition level coevolve (Williams *et al.* 2005). Indeed, if in response to the risk of sperm competition, males increase their ejaculate expenditure, they will not be able to produce many ejaculates (Parker 1998) which in turn will decrease the level of sperm competition in the population (Williams *et al.* 2005). Conversely, all else being equal, if males decrease the quantity of

sperm delivered to females they will be able to produce more ejaculates which will increase the level of sperm competition in the population (Williams *et al.* 2005). However, as pointed out by Parker and Ball (2005), the use of this correlative approach between sperm allocation and sperm competition does not generally invalidate the predictions of previous models.

ii) Raffles and roles models

As explained above, sperm competition is generally considered as a raffle where the probability of winning the competition is proportional to the number of sperm invested (Parker 1998). Considering two males in competition, the 'sperm raffle' between these two males can be fair when sperm from males 1 and 2 are equally favoured, or loaded when sperm from one male has an advantage over the sperm of the other male (Parker 1990a; Parker 1998). In some species, males might increase their chance of fertilization success by mating first, therefore sperm from the male mating in first position are favoured compared to sperm from males mating next (Dewsbury 1984; Parker 1998; Gomendio *et al.* 1998). Depending on the social structure and reproductive strategies of the species, males can have information about the role they occupy or not (Parker 1990a; Parker 1990b). Importantly, the role occupied by males can be random, which means that between all his copulations males will mate on average half of the time in a favoured role and half of the time in a disfavoured role (Parker 1990a). On the other hand, males might mate always in the same role for each of their reproductive events (non-random role situation). This is likely to be the case in species with a social hierarchy, where dominant males have priority access to females through intra- or intersexual competition (Andersson 1994; Modig 1996; see also **Section 1.4**).

The output of the models varies with the parameters described above (fair raffle versus loaded raffle; random roles versus non-random roles and information about roles versus no information about roles) (Parker 1990a; Parker 1998). However, in these models, I will be focusing on the scenario of a loaded raffle with males having information about their non-random roles as is the case where male roles and relative success differ predictably according to social dominance. In such situations, disfavoured males are predicted to invest more in sperm expenditure than favoured males (Parker 1990a; **Figure 1.3**). Tazzyman *et al.* (2009) suggested that variation between males' reproductive roles or strategies were more likely to be continuous but the output of their models were in accordance with Parker (1990a), with

males having a privileged access to females producing smaller or lower quality ejaculates (Tazzyman *et al.* 2009).

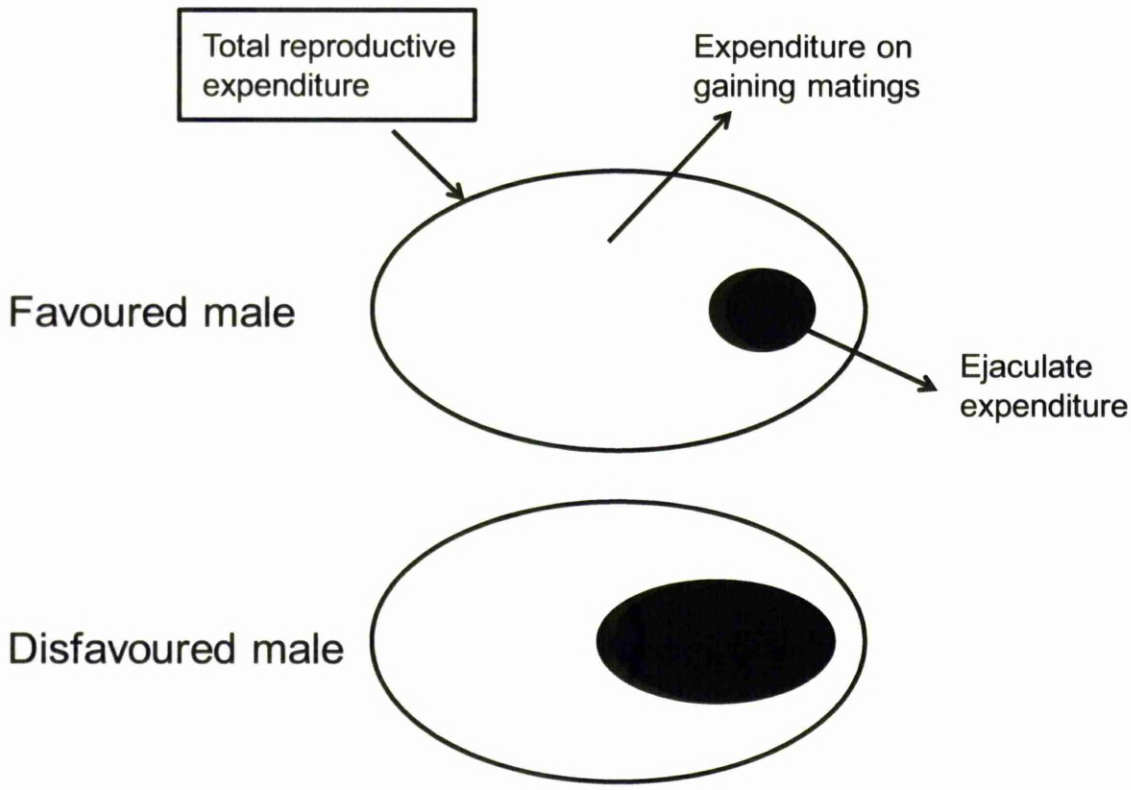

Figure 1.3 'Fried egg' diagrams representing the relative amounts that a male in a favoured role and a male in a disfavoured role should invest in reproduction when they are mating in a situation of non-random roles. Both males have the same total reproductive expenditure but males who mate in a disfavoured role are predicted to invest more in ejaculate expenditure than males who mate in a favoured role (modified from Parker 1998).

(d) Sperm allocation in mammals

To date, 'risk' and 'intensity' models have been tested in humans and in several species of rodents (e.g. Baker & Bellis 1993; Pound & Gage 2004; Ramm & Stockley 2007). In humans, a species with a low risk of sperm competition (Simmons *et al.* 2004), men who view images depicting a sperm competition situation (e.g. two males and a female) have as a result a higher percentage of motile sperm in their ejaculate than males who view images depicting a non-competitive situation (e.g. three females) (Kilgallon & Simmons 2005). However, intercultural differences in the risk of sperm competition (Simmons *et al.* 2004), reliability of

the data (Simmons *et al.* 2004) and weakness of control conditions in experimental design (review in Shackelford *et al.* 2005) often limit the conclusions that can be drawn from sperm competition studies in humans (see also Birkhead *et al.* 1997).

In a promiscuous rodent, the meadow vole (*Microtus pennsylvanicus*), males allocate sperm as predicted by the 'risk' and 'intensity' models. That is, when males perceive a risk of sperm competition (through olfactory cues), there is an increase in the number of sperm contained in the vas deferens (delBarco-Trillo & Ferkin 2007a) and the ejaculate (delBarco-Trillo & Ferkin 2004) independent of copulatory behaviour (delBarco-Trillo & Ferkin 2007b). The 'within-animal' design used in this study shows that each male increases his sperm investment when he perceives the odour of a potential competitor (delBarco-Trillo & Ferkin 2004). Moreover, male meadow voles increase their sperm investment when they face a 'high quality' competitor but not a 'low quality' competitor (i.e. food deprived male) (Vaughn *et al.* 2008), which reveals how males from this species can be plastic in their sperm allocation. The Norway rat (*Rattus norvegicus*) also increases sperm allocation when the risk of sperm competition increases (Pound & Gage 2004), although the variation in sperm investment is less pronounced in this species compared to the meadow vole. However, the sperm allocation pattern is different in house mice (*Mus musculus*) where males reduce their allocation of sperm (Ramm & Stockley 2007; but see Ramm & Stockley 2009b) and reduce the number of intromissions prior to their first ejaculation (Preston & Stockley 2006) when the risk of sperm competition is elevated through the presence of a rival male. This suggests that house mice might use a more cautious strategy, because males seem to reduce the risk of 'take-over' and to privilege a reduction of the ejaculation latency which can lead to a reduction in the number of sperm transferred (Preston & Stockley 2006; Ramm & Stockley 2007). Moreover, males who face a higher risk of sperm competition are more likely to start a second ejaculatory series that could compensate for the small number of sperm invested previously (Preston & Stockley 2006). These results also emphasize the importance of male copulatory behaviour in sperm allocation studies, since males respond to variation in sperm competition risk or intensity through adjustment in their copulatory behaviour that will have consequences for the number of sperm invested (Ramm & Stockley 2007). Although not well tested in mammals, predictions of the 'intensity models' have found support in the meadow vole where male sperm investment is maximal in the presence of the odour of one competitor but decreases when they face five competitors (delBarco-Trillo & Ferkin 2006).

'Raffles and roles' models have been far less tested in mammals, although a species of squirrel (*Spermophilus tridecemlineatus*) supports the prediction of the scenario where males mate in a random role (Schwagmeyer & Parker 1994). In species with social dominance relationships, where roles are non-random, favoured males are likely to be dominant and disfavoured males subordinate (see **Section 1.4**). In house mice, sperm from dominant males are more motile than sperm form subordinate males and this difference in sperm quality probably occurs after the establishment of social dominance (Koyama & Kamimura 1999; Koyama & Kamimura 2000) which suggests that the pattern of high sperm investment by subordinate males found in several taxa (e.g. Fu *et al.* 2001; Rudolfsen *et al.* 2006 in fishes, Froman *et al.* 2002; Cornwallis & Birkhead 2007 in birds) might not apply to mammals. Nevertheless, contrary to some other taxa, the quality of the ejaculate (i.e. sperm motility, sperm viability, review in Snook 2005) has not been well studied in rodents, at least in a sperm competition context.

1.4 Sexual selection and social dominance in mammals

(a) Social dominance in mammals

In many vertebrate species including mammals, social organization is based on dominance relationships (Smuts *et al.* 1987; Drews 1993; Andersson 1994). Drews (1993) reviewed 13 different definitions of dominance found in the literature and in this thesis I will define dominance relationships as a result of agonistic interactions between two individuals (Drews 1993). For males, these interactions generally occur for the purpose to have access to females (Andersson 1994) and when they are repeated, the consistent winner is the dominant and the consistent loser is the subordinate (Drews 1993; but see Dugatkin & Earley 2004 for the importance of the winner and loser effects). Therefore dominant males (i.e. more likely to win a contest) will generally have a privileged access to high quality territories and females (see below and **Section 1.4.b**). Dominance relationships are often based on body size and/or weight and age (Andersson 1994; Jacob *et al.* 2007), although in some species, weapons such as horns also play a role in male contests and thus in determining the outcome of male interactions (Andersson 1994).

In mammals, many species are polygynous and in some of these species, males control territories and/or access to females (Clutton-Brock 1989; McElligott *et al.* 2001; Coltman *et*

al. 2002; Waterman 2007). In elephant seals (*Mirounga* sp.) for example, dominant males are larger and defend large groups of females. They physically prevent subordinate males from having access to females (LeBoeuf & Peterson 1969; LeBoeuf 1974). In some rodent species, dominance can be age-dependent (Waterman 2007) such as in the beaver (*Castor canadensis*) (Busher 2007). In these species, scent marking is an important element in the establishment and the maintenance of social dominance and allows interactions between males without risk of injuries (review in Roberts 2007). In mice and bank voles[1], dominant males typically deposit numerous fine traces of urine whereas subordinate males deposit large pools of urine (Desjardins *et al.* 1973; Rozenfeld *et al.* 1987). Dominant males can also rapidly overmark the deposits of competitors (Hurst 1990). Moreover, investing in olfactory signalling is metabolically costly (Gosling *et al.* 2000) since it requires for example, a larger preputial gland (Kruczek 1997; Pohorecky *et al.* 2008).

(b) Effect of dominance on mating success

Dominant males generally have a higher mating and reproductive success (e.g. Dunbar & Dunbar 1977; Cowlishaw & Dunbar 1991; Coltman *et al.* 2002; Pelletier & Festa-Bianchet 2006; Wroblewski *et al.* 2009 but see Ellis 1995). For example, in rhesus macaques (*Macaca mulatta*), high ranking males, generally older, have higher reproductive success measured as the number of offspring sired in 6 years (Widdig *et al.* 2004). The higher reproductive success of dominant males can be explained by their advantage in intra and/or intersexual selection (Clutton-Brock & McAuliffe 2009; **Section 1.4.a**). Indeed, dominance can be a trait that is preferred by female mammals in mate choice, especially in rodents and primates (Clutton-Brock & McAuliffe 2009). For example, in primates such as savannah baboons (*Papio cynocephalus*), females benefit from mating with dominant males since they give better paternal care (Buchan *et al.* 2003; Clutton-Brock & McAuliffe 2009). In topi antelopes (*Damaliscus lunatus*), females actively compete to have access to the male at the central place in the lek, who generally are heavier (Bro-Jørgensen 2002) and in rodents, females generally prefer dominant males rather than subordinates and may often use the scent mark behaviour of males as an honest signal to base their choice on (review in Roberts 2007). Moreover, females can also benefit from mating with dominant males if dominance is heritable (e.g. Nol *et al.* 1996; Horne & Ylönen 1998).

[1] The special case of social dominance establishment in bank voles will be discussed in **Chapter 2**.

Interestingly, there is now evidence that mating with dominant males can sometimes be deleterious for female fitness. For example, in the red deer (*Cervus elaphus*) it might be costly for females to mate with high quality males since these males will sire daughters with significantly low fitness (Foerster *et al.* 2007). These potential costs of mating with high quality males were pointed out by Qvarnström and Forsgren (1998), who suggested that mating with dominant males might sometimes be suboptimal and that females should base their mate choice on the basis of the resources provided by males rather than on the male's social status *per se* (Qvarnström & Forsgren 1998). In the Soay sheep (*Ovis aries*), females mate multiply and dominant males have a higher mating success than subordinate males (Preston *et al.* 2001). Due to this higher mating rate, males are sperm depleted at the end of the rut period and females who will mate late with these males will suffer from sperm limitation (Preston *et al.* 2001). Such costs can even promote female harassment behaviour in the African topi antelope (*Damaliscus lunatus*) in order to prevent males from allocating sperm to other females (Bro-Jørgensen 2007).

1.5 Bank voles (*Myodes glareolus*)

(a) Ecology and population dynamics

The bank vole (*Myodes glareolus*; formerly *Clethrionomys glareolus*) is a palearctic rodent living in forests and fields (Raczyński 1983). Females are strongly territorial (Bujalska & Hansson 2000) and when the density of the population is high, the sexual maturation of young females is suppressed (Kruczek & Marchlewska-Koj 1986). Moreover, the size of male and female home ranges (foraging area) varies in relation to food availability (Bujalska & Saitoh 2000). Male home ranges are larger and overlap female home ranges during the breeding season (Bujalska 1973) which leads to the opportunity for aggression between males (Bujalska 1973). Individuals use scent marks to maintain their territories and larger males have larger home ranges (Bujalska & Saitoh 2000). Male bank voles disperse from their natal site (Gliwicz & Ims 2000) but unlike some other vole species, female bank voles also tend to disperse from their natal site even if the phenomenon and its occurrence is under debate in this rodent (Gliwicz & Ims 2000).

(b) Reproductive characteristics

The social structure and mating system of bank voles allow female multiple mating to occur regularly (Bujalska & Saitoh 2000), which is confirmed by molecular analysis such as DNA fingerprinting in wild populations (Ratkiewicz & Borkowska 2000, see **Section1.5.d**). The relatively large testis mass (controlled for body mass) of male bank voles is an adaptation to the high degree of female polyandry in this species (Ramm *et al.* 2005).

The range of the litter size varies from two to ten pups (Koivula *et al.* 2003) with an average litter size at 4.1 pups per litter (Mappes *et al.* 1995) which can slightly fluctuate during the breeding season (Bujalska 1983) and the quality of the environment (Koskela *et al.* 1998). The breeding season is between early April and late September (Bujalska 1983). Heavier bank voles at birth and weaning have faster sexual maturation and a better survival rate (Koskela *et al.* 1998). The adult weight of the male and the female vary between different populations (Yoccoz *et al.* 2000). Males attain sexual maturity at 2 months and females at 1.5 months (Bujalska 1983). In bank voles, female ovulation is induced by copulation (Clarke *et al.* 1970) which might have been responsible for the evolution of complex genitalia (see **Section 1.5.d**).

(c) Social dominance and mate choice

In bank voles, male dominance relationships play an important role in intra- and intersexual selection (Viitala 1977; Kruczek 1997). In low density populations, dominant males monopolize several females and easily exclude subordinates from reproduction whereas subordinates have more opportunities for reproduction when the density of the population increases (Bujalska & Saitoh 2000). Under laboratory conditions, male dominance status is assessed by various methods such as scent marks or agonistic interactions (Horne & Ylönen 1996; Kruczek 1997; **Chapter 2**). Dominant males are sometimes found to be heavier than subordinates (Kruczek & Styrna 2009) but not always (Klemme *et al.* 2006a) and the relationship between body mass and intensity of sexual selection is unclear in bank voles (Klemme *et al.* 2006b). As in other rodents such as house mice (Drickamer 1992), female bank voles show a preference for the odour of dominant males compared to subordinates (Kruczek 1997) and initiate copulations with dominant males in priority (Horne & Ylönen 1996). Olfactory signalling plays an important role in social communication in rodents (Hurst

2009) and cues of dominance are conveyed through urine in bank voles (Kruczek 1997; Marchlewska-Koj 2000). Olfactory signalling is costly in bank voles (Radwan *et al.* 2006) and dominant males invest more in signalling than subordinate males especially through their heavier preputial gland (Gustafsson *et al.* 1980; Łopuch & Radwan 2009). The higher level of testosterone found in dominant males (Kruczek 1997) is likely to be responsible for their bigger preputial gland (Gustafsson *et al.* 1980). Secretions from the preputial gland such as hexadecylacetate are mixed with urine and play a major role in social status signalling (Brink & Hoffmeyer 1984). By mating preferentially with dominant males, females might improve their fitness by having better quality offsprings (Kruczek & Zatorska 2008). Moreover, dominance appears to be a heritable trait in bank voles (Horne & Ylönen 1998; Oskanen *et al.* 1999) but only when the environment is stable (Mills *et al.* 2007).

(d) Sperm competition

Multiple mating is common in bank voles, and the number of multiply sired litters is estimated at 35.5 % in wild populations (Ratkiewicz & Borkowska 2000). The female propensity to mate multiply was confirmed by laboratory observations (Ratkiewicz & Borkowska 2000; Klemme *et al.* 2006a; see also Klemme *et al.* 2007a,b). Females increase their pregnancy probability by mating twice with either the same male or two different males (Klemme *et al.* 2007a) which suggests that female stimulation is important to initiate pregnancy (Klemme *et al.* 2007a). Polyandry in bank voles does not provide direct benefits such as an increase in litter size (Klemme *et al.* 2007a). However, in this species, females may reduce the risk of infanticide by mating multiply (Klemme & Ylönen 2010) and offspring of polyandrous females have better reproductive success than offspring from monandrous females (Klemme *et al.* 2008). Unfortunately, these last studies (Klemme *et al.* 2008; Klemme & Ylönen 2010) do not provide information on the dominance status of the males mated. However, in a study with control for male dominance status, it appears that when females mate with both dominant and subordinate males, dominant males have higher reproductive success than subordinate males, especially when they mate last (Klemme *et al.* 2006a). Female propensity to remate is not based on male mating status (Klemme *et al.* 2006a) and females will initiate copulations with both dominant and subordinate males. Females may benefit from mating with dominant males by weaning more pups per litter (Kruczek & Zatorska 2008 but see Klemme *et al.* 2006a) which does not seem to be mediated by a higher female investment in parental care (Oksanen *et al.* 1999). Also, the offspring of dominant

males may attain sexual maturity more quickly through having heavier testes or uteri (Kruczek & Zatorska 2008). However, as questioned by Klemme *et al.* (2006a), it is unknown if the better fertilization success of dominant males is due to sperm competition, female cryptic choice or both. In a recent study, Kruczek and Styrna (2009) found that dominant males have heavier testes and accessory glands than subordinates. This study also reveals that sperm quality is linked to male dominance status since dominant males have more motile, more concentrated and more viable sperm (Kruczek & Styrna 2009) which tends to suggest than dominant males might be better competitors in sperm competition. However, no information is given on the sperm allocation pattern, which can vary according to the sperm competition level (e.g. delBarco-Trillo & Ferkin 2006; Ramm & Stockley 2007), and it might be possible as predicted by theoretical studies (e.g. Parker 1990a; Tazzyman *et al.* 2009) that subordinates have higher ejaculate investment (see also Rudolfsen *et al.* 2006). Moreover, as explained above, stimulation during mating seems to have an important influence on female pregnancy initiation (Klemme *et al.* 2007a) and subordinate males could increase their investment in female stimulation. The copulatory behaviour of bank voles is generally described as a series of mounts and intromissions (without sperm transfer) leading to an ejaculation during which the male falls to one side carrying the female over with him (Milligan 1979; Klemme *et al.* 2007a). Males can ejaculate several times with the same female (Milligan 1979) even if information on the dynamics of multiple ejaculation series are for the moment limited due to the fact that many experiments under laboratory control are stopped after the male's first ejaculation (e.g. Klemme *et al.* 2006a). Information on stimulation by the male might be particularly relevant since female bank voles are induced ovulators (Clarke *et al.* 1970) and males have complex genitalia including a baculum (os penis) and penile spines (Milligan 1979). A copulatory plug is inserted by males at the end of an ejaculation but this plug does not prevent subsequent intromissions and copulations (Milligan 1979; see also **Chapter 5**).

1.6 Thesis overview

This thesis seeks to understand the process of sexual selection in the bank vole (*Myodes glareolus*) according to male dominance status and more precisely how sperm competition has influenced adaptations of dominant and subordinate males of this species. First, I will investigate the possibility of male mate choice in the bank vole, particularly in the context of inbreeding avoidance (**Chapter 2**). Next, since sperm competition can have consequences for

different aspects of the male phenotype, I will investigate the role of sperm competition on the evolution of male genitalia (**Chapter 3**), sperm allocation (**Chapter 4**) and sperm production (**Chapter 5**). In these three chapters, I will particularly focus on the dominant-subordinate relationship present in this species, which is fundamental to understand how males respond to the selective pressure of sperm competition.

It has been recently suggested that sperm competition could also have consequences on other aspects of the male phenotype such as brain size. This hypothesis will be tested using a comparative approach across mammalian species from different groups (**Chapter 6**). Finally, I will discuss the results and conclusions of each preceding chapters in order to draw a general picture of the influence of sperm competition in the bank vole and more broadly in mammals and vertebrates (**Chapter 7**).

Chapter 2: Male mate choice in the bank vole in relation to social dominance and level of sperm depletion

2.1 Chapter overview

Since inbreeding can result in reduced offspring fitness, female animals often avoid mating with close relatives. However, much less is known about how males respond to such mating opportunities. In theory, males may also avoid inbreeding under certain conditions, notably if alternative mating opportunities are available at low cost. For species where males differ in dominance status, dominant males typically have more mating opportunities than subordinates and may therefore be predicted to be less tolerant of inbreeding. More generally, sperm depletion following recent mating activity may influence inbreeding tolerance by constraining the ability of males to utilise available mating opportunities. Hence males may be predicted to be less tolerant of inbreeding following recent mating activity. I tested these predictions in a promiscuous rodent with clear male dominance relationships, the bank vole (*Myodes glareolus*). For males of known dominance status, I quantified behavioural responses in relation to sibling and unrelated females presented simultaneously under controlled experimental conditions, both prior to and after recent mating activity by the subject males. The behaviour of male bank voles appeared likely to promote inbreeding avoidance since they spent significantly less time in proximity to sibling females. However, my results do not provide clear evidence of a difference in behaviour according to male social status. Indeed, there was no significant difference in the strength of preference for unrelated females shown by dominant and subordinate males. Nonetheless, dominant males spent significantly more time in proximity to unrelated females compared to siblings whereas this difference in time visiting sibling and unrelated females was not significant for subordinate males, due to greater variation in this group. Contrary to predictions, following recent mating activity male bank voles did not show any significant difference in the time spend visiting unrelated and sibling females. These findings emphasize the importance of within-sex variation and associated

costs of reproduction in the study of inbreeding avoidance and, more generally, male mate choice.

2.2 Introduction

(a) Male mate choice

The study of male mate choice has generally received relatively little attention compared to female mate choice (Dewsbury 2005; Clutton-Brock 2007). This can be attributed to the influence of Darwin (1871) and Bateman (1948), who emphasized the importance in sexual selection of mate choice by females (Dewsbury 2005; Tang-Martinez *et al.* 2005). Emphasis on female mate choice and/or competition also follows from more recent insights regarding differential parental investment (Trivers 1972), and differences in potential reproductive rates between the sexes (Clutton-Brock & Parker 1992). Hence, females are expected to be the more choosy sex because they typically invest substantially more time and energy in the production and rearing of offspring compared to males (Andersson 1994; **Chapter 1**). Nevertheless, in two recent reviews, Clutton-Brock (2007; 2009) reconsiders the traditional view of sexual selection theory. Clutton-Brock (2007) emphasizes the fact that female competition for mates is probably widespread and not confined to sex-role reversed species. The presence of female competition is not incompatible with high male competition and the target of this competition is more frequently resources required for reproduction than mates (Clutton-Brock 2007). Nevertheless, in some cases, females can also compete for access to mates, especially when frequent mating induces sperm limitation in males, when multiple mating is advantagous (Clutton-Brock 2009; see also Bro-Jørgensen 2007). Moreover, in this reappraisal of sexual selection theory, Clutton-Brock (2009) suggests that added to this underestimated female competition, male mate choice is likely to be widespread and that its implication in sex role theory may be more important than previously thought. Indeed, theory predicts that male (or mutual) mate choice is expected to evolve if reproduction is costly for males (Dewsbury 1982a; Kokko & Monaghan 2001; Kokko & Johnstone 2002), if males have a high probability of attaining further reproductive opportunities (Owens & Thompson 1994; Johnstone *et al.* 1996) or if there is large variation in female quality (Burley 1977; Parker 1983; Bonduriansky 2001). This last point is critical since important differences in female fecundity within species are widespread (Clutton-Brock 2007). There is indeed some

empirical support for these predictions (e.g. Hill 1993 in birds; Amundsen & Forsgren 2001 in fishes), though relatively few studies have been conducted in mammals (Craig *et al.* 2002; Preston *et al.* 2005; but see **Section 2.2.c**). It is also important to note that male mate choice can sometimes be cryptic when males adjust the quantity of sperm allocated to females according to their quality (Cornwallis & Birkhead 2006; Rubolini *et al.* 2006). For instance in red junglefowl (*Gallus gallus*), males invest more sperm in MHC-dissimilar female than in MHC-similar female (Gillingham *et al.* 2009) since MHC-dissimilar female are more likely to be genetically unrelated (see **Section 2.2.c**). Ultimately, their offsprings would show a higher resistance to parasites (Milinski 2006).

(b) Female quality

When males are choosy, theory predicts that they will choose females of greatest reproductive value, so as to maximize the number of surviving offspring (Parker 1983). In a wide range of species, males choose females on the basis of their size, since this trait is often a good proxy for fecundity and thus of female quality (Wong & Jennions 2003; Herdman *et al.* 2004; Bel-Venner *et al.* 2008; Zahradnik *et al.* 2008; see also Teng & Zhang 2009). Other relevant traits in this context could be female mating history (Dewsbury 1981; Orrell & Jenssen 2002) or age (Muller *et al.* 2006; Parga 2006). For example, in the lizard *Anolis carolinensis*, males prefer novel females since these are less likely to have recently mated compared to resident females (Orrell & Jenssen 2002). In chimpanzees (*Pan troglodytes*), males prefer mating with older females which may be due to the mothering experience of these females or their higher genetic quality (Muller *et al.* 2006). A further factor that might influence a female's perceived value as a mate is her degree of relatedness to the male, because mating between close relatives generally leads to inbreeding depression (Charlesworth & Charlesworth 1999; Roff 2002; Charlesworth & Willis 2009).

(c) Inbreeding avoidance

Inbreeding can result in reduced offspring fitness due to an increase in the expression of homozygous deleterious recessive alleles (partial dominance) or to the loss of heterozygous benefits (overdominance) (review in Charlesworth & Willis 2009). Inbreeding avoidance behaviour may therefore be favoured to maximise reproductive fitness, despite potential

inclusive fitness benefits of mating with relatives (Parker 1979; Lehmann & Perrin 2003). There is widespread evidence that female animals often favour unrelated partners, with preferences expressed via pre- and/or post-copulatory processes (Pusey & Wolf 1996; Tregenza & Wedell 2000; Pizzari *et al.* 2004; Hoffman *et al.* 2007). In contrast, because males typically have higher potential reproductive rates than females, and encounter sexually receptive mates less frequently, they are generally expected to be more tolerant of inbreeding (Parker 1979; Parker 2006). However, males may also exhibit inbreeding avoidance behaviour under certain circumstances, for example if encounter rates with sexually receptive females are relatively high and / or search costs are low (Parker 1979, 1983, 2006; Koko & Ots 2006). To date, there have been relatively few empirical tests of male inbreeding avoidance behaviour and findings vary across taxa. For example, male cockroaches (*Blatella germanica*) are reported to preferentially court non-sibling females over siblings (Lihoreau *et al.* 2008) but no difference was found in the copulatory behaviour of male guppies (*Poecilia reticulata*) according to female relatedness (Pitcher *et al.* 2008). Similarly, there is evidence that male Indian meal moths (*Plodia interpunctella*) invest more sperm in copulations with unrelated females than with related females (Lewis & Wedell 2009), whereas red junglefowl males (*Gallus gallus*) instead invest more sperm when mating with related females (Pizzari *et al.* 2004; but see also Gilligham *et al.* 2009). In mammals, evidence of male mate choice based on female relatedness is scarce (Pusey & Wolf 1996), but male dispersion could have evolved to avoid inbreeding depression (Lehmann & Perrin 2003; Ishibashi & Saitoh 2008). However, in mammals there are now few evidences that males show a preference for MHC dissimilar partners (e.g. Yamazaki *et al.* 1988; Beauchamp *et al.* 1998 in lab mice or Wedekind & Furie 1997 in humans) since individuals with the same MHC alleles are likely to be related (Penn & Potts 1999; Gilligham *et al.* 2009).

The likelihood of successful copulations occurring between close relatives will also be determined by factors influencing within-sex variation in inbreeding tolerance. For example in common lizards (*Lacerta vivipara*), female tolerance to inbreeding is age-dependent, with younger and older females more tolerant than females of intermediate age (Richard *et al.* 2009). Variation in the degree of inbreeding tolerance may also be expected in males, although male mate preferences are generally less well studied compared to those of females (Dewsbury 2005). For example, males that are able to monopolise the best areas for access to mates, or are preferred by females as mates, are likely to experience relatively low search

costs and high encounter rates with sexually receptive females (Cowlishaw & Dunbar 1991; van Noordwijk & van Schaik 2004). Such males may therefore be expected to be more discriminating compared to less competitively successful males (Fawcett & Johnstone 2003, Härdling *et al.* 2008). Hence, in species where male dominance correlates with access to sexually receptive females, dominant males are expected be less tolerant of inbreeding than subordinates because they generally have lower search costs and more mating and / or fertilization opportunities (Parker 2006).

Inbreeding tolerance may also vary adaptively for individual animals according to conditions at the time of mating (e.g. Richard *et al.* 2009). Particularly in species with promiscuous mating systems, sperm depletion can potentially influence male mate choice decisions (Dewsbury 1982a; Preston *et al.* 2003; Härdling *et al.* 2008). Despite some evidence that sperm depletion can interact with male mate choice (Sæther *et al.* 2001; Byrne & Rice 2006; Lemaître *et al.* 2009), the potential consequences of this for inbreeding avoidance behaviour remain to be explored. Here, I predict that because sperm depleted males are likely to experience an elevated cost of reproduction (since they are constrained in the number of females they can mate with), they should be less likely to invest in copulations with related females where alternative mating opportunities are available.

In the present study, I aim to explore these ideas in a small mammal, the bank vole, *Myodes* (formerly *Clethrionomys*) *glareolus*. There is evidence that inbreeding can induce a fitness cost in this species, since Kruczek (2007) found that only 28% of females gave birth after mating with a brother, compared to 93% after mating with an unrelated male. Within the promiscuous mating system of bank voles, there are typically clear dominance relationships among males (Viitala 1977; **Chapter 1**), and dominant males achieve higher reproductive success than subordinates in both pre- and post-copulatory competition (Klemme *et al.* 2006a; Kruczek & Zatorska 2008). Dominant males may therefore be expected to have a lower inbreeding tolerance than subordinate males, due to their greater fertilization opportunities (Parker 2006). More generally, both dominant and subordinate males may be less tolerant of inbreeding when they are sperm depleted, since in this case mating opportunities are limited by their own sperm reserves (Byrne & Rice 2006; Härdling *et al.* 2008). The present study is designed to test each of these predictions experimentally for the first time in a mammal.

2.3 Methods

(a) Subjects

Bank voles used in the experiments were the adult F1 offspring of 29 wild-caught individuals (15 males and 14 females) trapped in Cheshire (UK) between January and May 2007. After weaning, animals were housed with siblings of the same sex in MB1 cages (40 X 23.5 X 20 cm, North Kent Plastic Cages Ltd., UK) containing substrate (Corn cob Absorb 10/14 substrate) and paper-wool bedding material (hereafter called bedding). Females were housed individually for the duration of the experiment (25 weeks) to avoid mixing their odours prior to experimental tests. Food and water were provided *ad libitum* (LabDiet 5002). Animals were maintained on a reversed photoperiod (light: 16h, dark: 8h, lights on at: 5 pm), and at a temperature of 21 ± 1°C. For purposes of identification, male subjects were PIT tagged.

(b) Social dominance establishment

In bank voles, several methods are generally used to assess male social status. Sometimes two males and a female are released in an arena at the same time and the first male who mates with the female is considered to be the dominant one (e.g. Oksanen *et al.* 1999; Mills *et al.* 2009). This method assumes that there is a strong advantage for males to mate with the female first, although dominant males seem to have a greater advantage over subordinate males when they mate in second position (Klemme *et al.* 2006a). Aggression tests are also frequently used to determine male social status in bank voles (e.g. Kruczek 1997; Łopuch & Radwan 2009). Basically, two males are placed together in an arena for a small amount of time and the number of aggressive and submissive behaviours of each male counted (this procedure being generally repeated over a few days). The male that shows the highest aggressive score is considered to be dominant (e.g. Kruczek & Zatorska 2008). Nevertheless, results obtained from this technique are sometimes contradictory (see Jones & Nowell 1989; Kruczek & Zatorska 2008; Łopuch & Radwan 2009). Moreover this technique involves non-negligible stress for the animals as well as possible injuries. To avoid direct aggressive contact between males, in this study I decided to follow a third technique based on the scent marking behaviour of males (e.g. Rozenfeld *et al.* 1987; Horne & Ylönen 1996; Klemme *et al.* 2006a; Roberts 2007). Pairs of unrelated males were set up in MB1 cages divided in two by a mesh

barrier with one male of each pair housed in each part of the cage (i.e. in a 20 x 11.75 x 20 cm area) over a period of 3 to 5 weeks. This separation allowed continuous olfactory and visual contact between males while they were housed together until the end of the experiment (**Figure 2.1**).

To assess scent marking behaviour, both males from a pair were transferred to clean benchkote-lined MB1 cages (again divided in two by a mesh barrier) and left for 30 min during the dark phase period. Then, scent marks were scanned using Bio-rad Fluor-S^{TM} MultiImager (QuantityOne software: 12sec exposure, 530DF60 Filter, UV light source Epi illumination, high resolution mode). The social status of the male was assessed based on the criteria identified by Rozenfeld and Rasmont (1991; but see also Horne & Ylönen, 1996). Specifically, thin streaks of urine throughout the arena are characteristic of dominant males. By contrast, subordinates deposit large puddles of urine, especially in corners of the arena (Rozenfeld *et al.* 1987; Rozenfeld & Rasmont 1991; Klemme *et al.* 2006a; **Figure 2.2**) although sometimes subordinate males can also stop marking (Desjardins *et al.* 1973; Rozenfeld & Rasmont 1991). Bedding from an unrelated female (approximately 13g) was added once prior to the first collection of scent marks from each male pair to increase male competitiveness (Kruczek 1997). Male roles as dominant and subordinate were assigned when a clear pattern of differences in scent marking between paired males (e.g. **Figure 2.1**) was obtained on three consecutive days (with collection of scent marks separated by at least 24 hours). Then, males were kept paired until the end of the experiment (see **Section 2.3.c**). If no clear dominant-subordinate pattern could be identified within a pair, males were housed back in their original cages or paired with a new unrelated male.

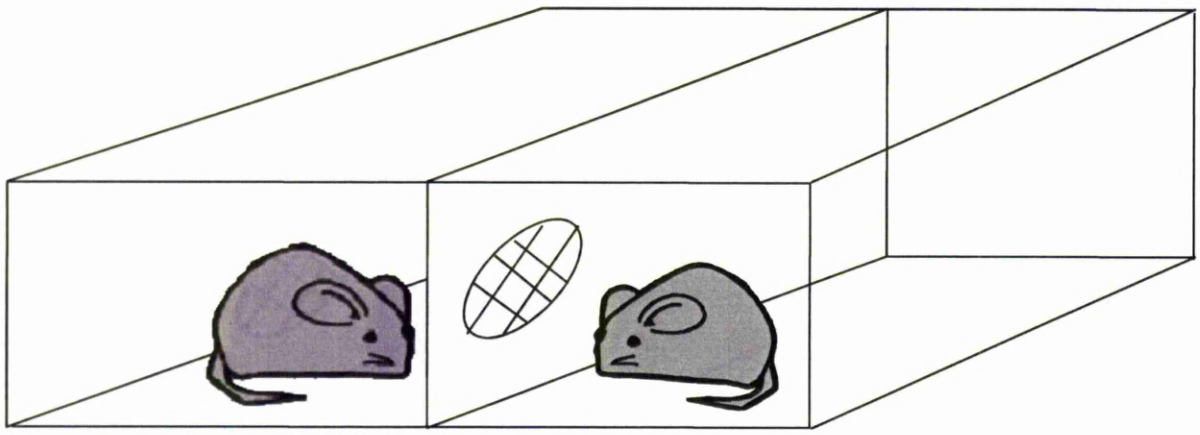

Figure 2.1 Diagram representing the experimental design to establish dominant-subordinate relationships. MB1 cages are divided in two by a mesh barrier with one male of each pair housed in each part of the cage (i.e. in a 20 x 11.75 x 20 cm area). The separation allows continuous olfactory and visual contact between males while they are housed together.

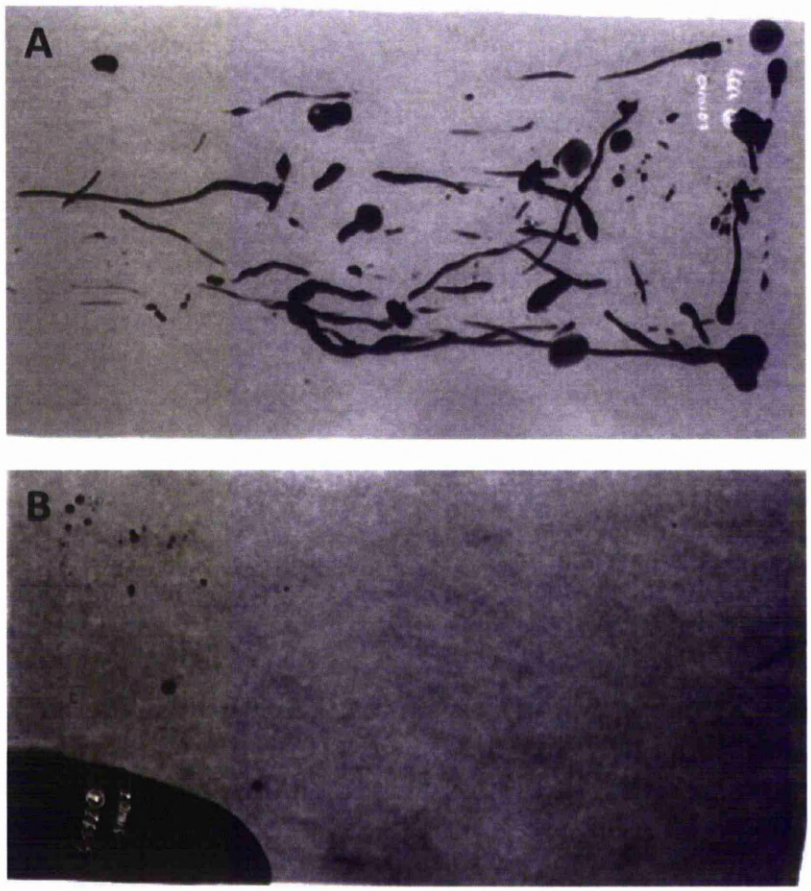

Figure 2.2 Scent marks from a dominant (A) and a subordinate (B) male bank vole. Typically, dominant males deposit thin streaks of urine throughout the arena whereas subordinate males deposit large pool of urine, especially in corners of the arena.

(c) Quantifying inbreeding avoidance behaviour

To assess male inbreeding avoidance behaviour, I used a choice chamber consisting of three interlinked MB1 cages. A central (neutral) cage was linked via tunnels (3 cm diameter) to two adjacent cages. Each adjacent cage was divided in two by a mesh barrier, forming one large central chamber and two smaller outer chambers. During behavioural tests, subject males were placed in the central chamber with a female in each of the two outer chambers. This allowed the male visual, auditory and olfactory contact with potential mates. The day before the start of the experiment, males were habituated to this experimental apparatus for 30 mins, with female bedding (approximately 30g) in the two outer cages in order to expose each male to female odours. Female odour samples used for this prior exposure were taken from the stock population rather than from females that the male would encounter in the experiment.

To test if male bank voles show more persistent interest in unrelated females than related females, and if their responses differ according to dominance status, I offered 28 males of known dominance status simultaneous access to a sister and an unrelated female. Dominant and subordinate males from the same pair were tested with the same females, such that one female was related to the dominant and one female was related to the subordinate. In most cases (22/28 males), sisters were full siblings selected from a different litter from the subject male (hereafter called related unfamiliar), whereas for the remaining 6 males (due to limited availability of unfamiliar siblings), sisters were taken from the same litter (hereafter called related familiar). In such cases, males and females were housed together until weaning (approximatively 22 days). Females (n = 32) were used with one or two pairs of males but when they were used twice, the paired female was different each time. I recorded male behaviour for a period of 30 minutes after the male had visited each female cage once and came back into the central cage. DVD recordings were analysed to quantify the time that subject males spent in each female's cage and the number of visits to each cage.

To test for an effect of male sperm depletion on inbreeding avoidance behaviour, and to investigate if any such effect differs according to male dominance status, I repeated the same test as above (with the same subjects and test females) but in this case males were tested shortly after mating to satiety with a third female (unrelated to the male and both females in the preference test; see **Chapter 4**). I identified an ejaculation as the last in a series if no mount or intromission occurred for 30 minutes afterwards (Stockley & Preston 2004). The average duration of a copulation series was approximately 80 minutes (see **Chapter 4**). During this time, males typically ejaculated three times before reaching satiety (mean ejaculation number ± s.e.m = 3.24 ± 0.22), which is sufficient to significantly deplete sperm reserves in other vole species (Pierce *et al.* 1990). Unfortunately, 4 subjects died between the two tests and the sample size was consequently reduced to n = 24 in this experiment. In each case, this second test was conducted at least 1 day (but maximum 2 weeks) after the first test, and between 45 and 60 minutes after completion of the subject male's last ejaculation. In addition to the main test for an effect of female relatedness on male behaviour, I also tested *a posteriori* for putative effects of female age or weight on male behaviour, since these might also influence male reproductive success (e.g. Muller *et al.* 2006; Xu & Wang 2009). Females used in these tests were between 61 and 319 days old and their weight between 14.89g and 25.21g.

(d) Statistical analysis

Normality of the data was tested by Kolmogorov-Smirnov tests and when necessary log-transformations were applied to improve it. To test simultaneously for an effect of female relatedness and male dominance status on the time that males spent in each female cage, I runed a 2 x 2 ANOVA including relatedness (unrelated female versus related female) as a within-subjet factor and the dominance status (dominant males versus subordinate male) as a between-subject factor. Then, comparisons between the time spent by males in unrelated and related female cages or between the number of visits to theses cages were performed using paired t-tests. To control for an effect of female age and weight on the male preference, I used a generalized linear model (GLM) using the time difference spent by males between the two female cages as the dependant variable, the female relatedness as a factor and the age and weight differences of the two females presented as covariates. All tests were two tailed and were conducted using SPSS 16.0. Data are presented as means ± s.e.m and differences were regarded as statistically significant at $P < 0.05$.

2.4 Results

(a) Male inbreeding avoidance behaviour and social status

Overall, males spent less time visiting related females compared to unrelated females (related: $\overline{X}$ = 525.9 ± 62.1s; unrelated: $\overline{X}$ = 882.6 ± 71.1s; t_{27} = -2.50, P = 0.02), although there was no difference in the number of visits to the female cages (related: $\overline{X}$ = 10.6 ± 1.0 visits; unrelated: $\overline{X}$ = 10 ± 1.3 visits; t_{27} = -0.76, P = 0.45). The tendency of male bank voles to spend less time visiting related females appears to be independent of familiarity (related-familiar: $\overline{X}$ = 414.1 ± 69.8s; related-unfamiliar: $\overline{X}$ = 556.3 ± 76.1s), since when both variables 'relatedness' and 'familiarity' are analyzed simultaneously in a GLM, only 'relatedness' explains the time that males spent in each cage (relatedness: $F_{1,27}$ = 7.04, P < 0.02; familiarity: $F_{1,27}$ = 1.74, P = 0.20).

I next examined differences in male behaviour according to social status. When I took in account in the same model the effects of female relatedness and male dominance to explain the time spent by males in each cage, I found similarly as above a significant effect of female

relatedness ($F_{1,26}$ = 6.08; P = 0.02). However, this effect appears to be independent of the male social status ($F_{1,26}$ = 0.09; P = 0.67). Indeed, dominant males spent significantly less time visiting related females (related: $\overline{X}$ = 509.6 ± 88s; unrelated: $\overline{X}$ = 857.5 ± 85s; t_{13} =2.32, P = 0.04) whereas although subordinate males tended to spend more time in unrelated female cages, the difference is not significant (related: $\overline{X}$ = 542. 2 ± 90.9s; unrelated: $\overline{X}$ = 787.7 ± 116.6s; t_{13} = 1.30, P = 0.22) (**Figure 2.3**). However, the strength of the preference for the unrelated female (measured as the difference between the time spent by males in the cage of the unrelated female and the cage of the related female) did not differ between dominant and subordinate males (dominant: $\overline{X}$ = 347.9 ± 149.5s; subordinate: $\overline{X}$ = 245.5 ± 188.6s; t_{26} = 0.43, P = 0.67) suggesting as above that the preference for the unrelated female is independent of male social status. The number of visits between the two cages did not differ significantly either for dominant males (related: $\overline{X}$ = 10.6 ± 1.3; unrelated: $\overline{X}$ = 10.6 ± 2.0; t_{13} = -0.06, P = 0.95) or subordinate males (related: $\overline{X}$ = 10.5 ± 1.7; unrelated: $\overline{X}$ = 9.5 ± 1.5; t_{13} = -1.27, P = 0.22). There was no difference in body mass between dominant and subordinate males (dominants: $\overline{X}$ = 23.5 ± 0.7g; subordinates: $\overline{X}$ = 23.2 ± 0.9g; t_{26} = 0.76, P = 0.77).

Finally, I tested *a posteriori* for additional putative factors that might affect male behaviour (female age and body mass). Confirming the results described above, the only factor found to have a significant effect on the time that males spent visiting female cages was the relatedness of the female (GLM: relatedness: $F_{1,27}$ = 5.20, P = 0.03; female age: $F_{1,27}$ = 1.60, P = 0.21; female body mass: $F_{1,27}$ = 0.07, P = 0.79).

(b) Effects of sperm depletion

Following sperm depletion, there was no significant difference in the time spent visiting related versus unrelated females for all males combined (related: $\overline{X}$ = 569.4 ± 67.2s; unrelated: $\overline{X}$ = 668.1 ± 75.7s; t_{23} = 0.79, P = 0.44) (**Figure 2.4**). Again, no difference was found in the number of visits according to female relatedness in this test (related: $\overline{X}$ = 13.3 ± 1.7; unrelated: $\overline{X}$ = 13.6 ± 1.5; t_{23} = -0.70, P = 0.48) or in subsequent tests (results not shown). No significant effect of relatedness, female age or female body mass on the time that males spent in female cages was found when these factors were analysed simultaneously in

the same model (GLM: relatedness: $F_{1,23} = 0.65$, $P = 0.43$, female age: $F_{1,23} = 0.30$, $P = 0.86$, female body mass: $F_{1,23} = 0.17$, $P = 0.68$). That is, neither the relatedness, age or body mass of potential mates appeared to have any effect on the time that sperm-depleted males spent visiting them.

When I tested simultaneously for an effect of female relatedness and male dominance status, I found similarly as above that males did not spend significantly more time in one of the female cage ($F_{1,22} = 0.59$; $P = 0.50$) and that male preference was not influenced by the male social status ($F_{1,22} = 0.003$; $P = 0.96$). Indeed, there was no significant difference in the time spent visiting related versus unrelated females for either group (dominant males: related: $\overline{X}$ = 632.1 ± 101.0s, unrelated: $\overline{X}$ = 724.8 ± 100.1s, $t_{13} = 0.50$, $P = 0.63$; subordinate males: related: $\overline{X}$ = 481.6 ± 74.9s; unrelated: $\overline{X}$ = 588.8 ± 117.3s, $t_9 = 0.66$, $P = 0.52$). Hence, although dominant males spent less time visiting related females when they were sexually rested (see above), the same effect was no longer evident following recent mating activity.

For all subjects combined, the total number of visits to female cages was significantly higher when males were sperm depleted compared to when they were sexually rested (sperm depleted: $\overline{X}$ = 27.1 ± 3.0, sexually rested: $\overline{X}$ = 21.0 ± 2.5, $t_{23} = -2.09$, $P < 0.05$), although there was no significant difference in the total time spent in the two female cages according to recent male sexual activity (sexually rested: $\overline{X}$ = 1344.5 ± 71.7s; sperm depleted: $\overline{X}$ = 1237.5 ± 69.7s; $t_{23} = -1.32$, $P = 0.20$).

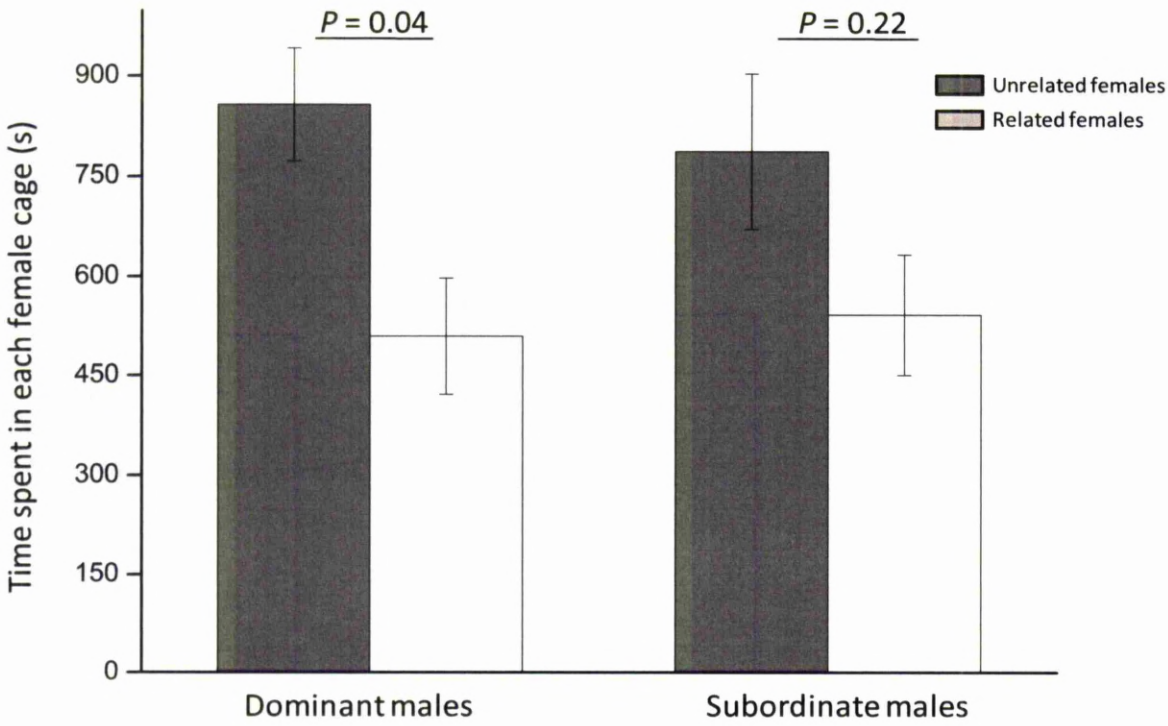

Figure 2.3 Mean time (in seconds) ± s.e.m spent by dominant and subordinate males in the cages of unrelated and related females. Dominant males spent significantly less time visiting related females (related: $\overline{X}$ = 509.6 ± 88s; unrelated: $\overline{X}$ = 857.5 ± 85s; t_{13} =2.32, P = 0.04) whereas although subordinate males tended to spend more time in unrelated female cages, the difference is not significant (related: $\overline{X}$ = 542. 2 ± 90.9s; unrelated: $\overline{X}$ = 787.7 ± 116.6s; t_{13} = 1.30, P = 0.22). However, the male preference for unrelated females appears to be independent of the social status ($F_{1,26}$ = 0.09; P = 0.67).

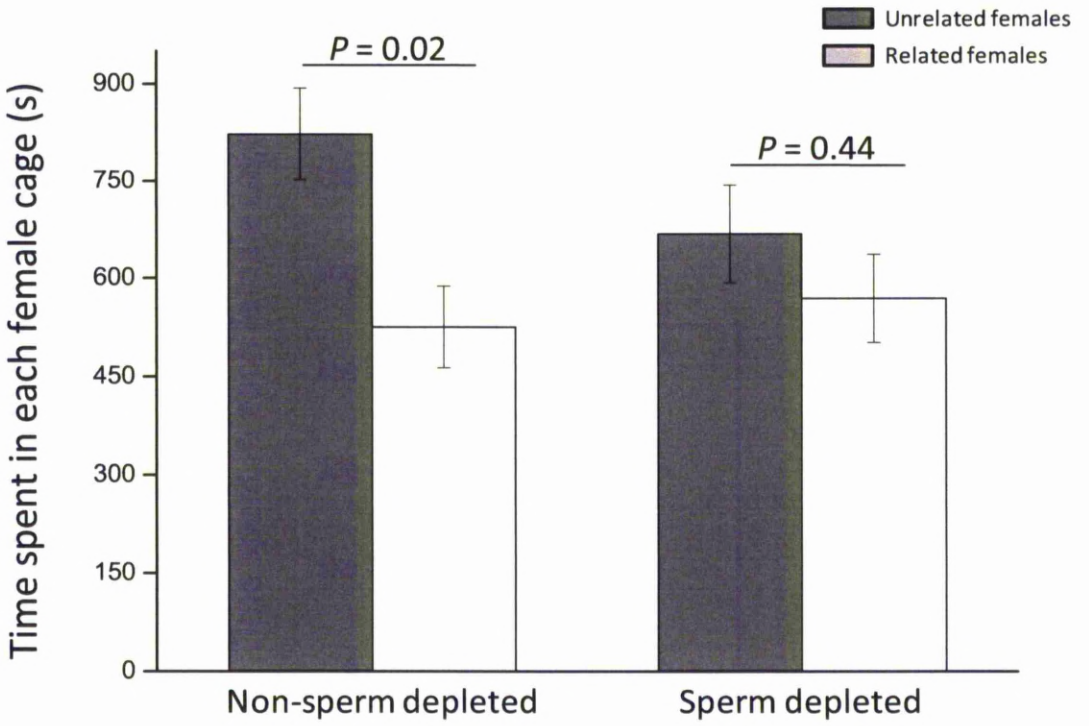

Figure 2.4 Mean time (in seconds) ± s.e.m males spent in the cages of unrelated and related females before they mated to satiety with a female (non-sperm depleted) and after (sperm-depleted). When they were non-sperm depleted, males spent significantly more time with unrelated females (t_{27} = -2.50, P = 0.02) but this preference disappeared when they were sperm-depleted (t_{23} = 0.79, P = 0.44).

2.5 Discussion

When male bank voles were offered simultaneous access to sibling and unrelated females, they spent significantly less time in proximity to sibling females. These findings concur with results from Kruczek & Gołas (2003) who show that male bank voles switch their preference for their mother to a preference for unrelated females when they become sexually mature (Kruczek & Gołas 2003). Assuming that the time spent in proximity to females reflects male sexual interest, our findings indicate that male bank voles may be less likely to pursue copulation attempts with sisters than with unrelated females. Although inbreeding avoidance behaviour has generally been studied from the point of view of the female, a few other studies suggest that adult male rodents show a preference toward unrelated females (Barnard & Fitzsimons 1988; Solomon & Rumbaugh 1997). For instance, in the common vole (*Microtus arvalis*), adult males show a preference for the odour of unrelated females compared the odour of sibling females (Bolhuis *et al.* 1998).

The findings of this study add to growing evidence of kin recognition in mammals (e.g. Cheetham *et al.* 2007; Mateo 2009), and are consistent with results of previous studies demonstrating that bank voles discriminate between related and unrelated individuals (Kruczek & Golas 2003; Kruczek 2007). Various kin recognition mechanisms have evolved across taxa (e.g. Parr & de Waal 1999; Lihoreau & Rivault 2009) but in many rodents social communication (including kin recognition) relies predominantly on chemical signals conveyed through urine (Hurst 2009). Radwan *et al.* (2008) suggested that in bank voles major histocompatibility complex (MHC) molecules could be implicated in kin discrimination. However, in house mice (*Mus musculus*), recent evidence indicates that a family of lipocalin proteins known as major urinary proteins (MUPs) play a more significant role than MHC in kin recognition (Sherborne *et al.* 2007) and other aspects of social and sexual behaviour (Cheetham *et al.* 2007; Thom *et al.* 2008; Hurst 2009). Lipocalins are present in bank vole urine (Turton 2007), but whether they have a similar function to that described in house mice remains to be determined.

In the present study, I did not find a significant difference in the strength of the preference for unrelated females between dominant and subordinate male bank voles, suggesting that these males may show inbreeding avoidance behaviour regardless of their social status. However, unlike dominant males, subordinate males did not spent significantly more time in the vicinity

of unrelated females. This is most likely explained by a greater variance in investigation behaviour of subordinate males that appears to be larger than for dominant males. Theoretical predictions suggest that in situations of high male-male competition, disfavoured competitors should be less choosy than favoured competitors (Fawcett & Johnstone 2003; Härdling *et al.* 2008) and these predictions have recently found support in the threespine stickleback (*Gasterosteus aculeatus*) where males show a preference for larger females but when male-male competition increases, only males in good-condition continue to prefer larger females whereas males in poor-condition become indiscriminate (see Candolin & Salesto 2009). However, evidence for variations in inbreeding tolerance behaviour according to differences in male social dominance remains for the moment limited.

Sperm depletion is likely to increase mating costs (Dewsbury 1982a; Härdling *et al.* 2008) and males are expected to be more stringent in mate choice when these costs increase (Kokko & Monaghan 2001; Kokko & Johnstone 2002; Byrne & Rice 2006). However, these results suggest that male bank voles may be more tolerant to inbreeding under such circumstances since, in the behavioural tests involving recently mated males, there was no significant preference for unrelated females. One possibility to explain this result could be that after copulating to satiety, males experience a refractory period during which sexual motivation is reduced. This explanation seems unlikely, however, because I did not observe any decrease in the amount of time males spent in the two female cages when comparing 'non sperm depleted' and 'sperm depleted' males, and males were in fact more active in moving between female cages after having mating to satiety. Hence it appears that male bank voles were still interested in females after mating with a different partner but the reason why males were apparently less discriminating with respect to female relatedness following sperm depletion remains unclear.

These results also suggest that in bank voles, possible inbreeding avoidance behaviour influenced by male sperm reserves could have implications for understanding variation in sexual conflict over mating decisions (see Parker 1979, 2006). That is, assuming that female bank voles are typically resistant to copulation attempts by closely related males due to the high fitness costs of inbreeding in this species (Kruczek 2007), the occurrence of sexual conflict over mating decisions between relatives would be likely to vary according to male recent mating activity. Hence, it seems that non sperm-depleted males might be more likely to

concur with female mating decisions to avoid inbreeding, whereas conflict over mating may be more likely when males suffer from sperm depletion. In the context of the present study it is also important to note that females benefit from mating with non sperm-depleted individuals (Preston *et al.* 2001; Arnqvist & Rowe 2005; see also Bro-Jørgensen 2007). Hence a potential proximate explanation for our findings is that male bank voles are responding to signals of female sexually receptivity, and that females actively solicit more attention from males who have not mated recently. However, recent evidence suggests that receptive female bank voles nearly always accept copulation attempts (Klemme *et al.* 2007a), perhaps due to benefits of multiple mating (Klemme *et al.* 2008; Klemme & Ylönen 2010) and more importantly it seems unlikely that females would be able to assess male mating status in this experiment.

Male bank voles in this study showed no preference for females as a function of either their body mass or age, which contrasts with recent findings such as the preference for larger females in the fiddler crab (*Uca mjoebergi*) (Reading & Backwell 2007) or the preference for older females in chimpanzees (*Pan troglodytes*) (Muller *et al.* 2006). However, whilst such traits may often reflect female fecundity (and therefore reproductive value), and thus could form the basis for adaptive male mate choice (Bonduriansky 2001; Xu & Wang 2009), in bank voles there is no positive correlation between female body weight and the litter size (Koskela *et al.* 1998; **Chapter 4**) and no report of a decrease in female reproductive success with age. However, it cannot be excluded that males might need some direct contact with females to assess these quality clues, especially body mass.

2.6 Conclusion

I conclude that male bank voles exhibit behaviour that is likely to promote inbreeding avoidance, but that there is no clear difference in such behaviour according to male dominance status. However and contrary to theoretical predictions, males appear more tolerant to inbreeding when they are sperm-depleted. These results imply that potential benefits of mate choice may vary considerably among males. I suggest that such variability should be taken into account in future studies of inbreeding avoidance behaviour, not least because it might help to explain common mismatches between theoretical predictions and empirical evidence, as pointed out by Kokko and Ots (2006).

Chapter 3: Genital morphology and allometry in the bank vole

3.1 Chapter overview

In mammals, genitalia can be complex, including the presence of a baculum (os penis) and keratinized spines at the surface of the penis. Although it is now well accepted that post-copulatory sexual selection can be responsible for the rapid evolution of genitalia, the exact process involved in this evolution is still unclear (e.g. sperm competition, female cryptic choice, sexual conflict). Similarly many hypotheses concerning the function of elaborated genitalia have been proposed but none have yet found unequivocal support.

Bank vole genitalia are particularly interesting since males have both a baculum and penile spines. In this species, females mate multiply with dominant and subordinate males in the same reproductive bout but dominant males perform better than subordinate males in terms of reproductive success. However, it is still unknown if the higher fitness of dominant males is achieved through an advantage in sperm competition, female cryptic choice or both. In the present study, I investigated for the first time in mammals if genital morphology differs according to male social status by comparing the baculum (width and length) and spinosity (spines length, spines density, percentage of the penis covered by spines) between dominant and subordinate males. Since models of sexual selection generally predict that sexually selected traits should exhibit positive allometry and a high coefficient of phenotypic variation, I also tested for positive allometry of the baculum and spine dimensions in bank voles.

The results showed that baculum basal width was significantly larger in dominant males than in subordinates, whereas the length of the baculum did not significantly differ according to male social status. Similarly, I did not find differences between dominant and subordinate males in the three parameters of spinosity investigated. Therefore, dominant males might be favoured by cryptic female choice if an enlarged baculum provides more stimulation to the female during mating. Among all the baculum and spines traits investigated, only the baculum

basal width shows positive allometry associated with a high coefficient of phenotypic variation, suggesting that this trait is likely to be influenced by sexual selection.

3.2 Introduction

(a) Structure and diversity of genitalia in mammals

From insects to mammals, male genitalia present a large variety of phenotypes (Eberhard 1985) which constitute a powerful tool for taxonomists to identify species (Hooper & Musser 1964; Anderson 2000). In mammalian taxa, male genitalia can be complex, sometimes including a baculum (os penis) located inside the penis (Burt 1960). A baculum is found in bats, carnivores, insectivores, primates and rodents (Patterson & Thaeler 1982). Some mammals also bear keratinized spines at the surface of their penis (Eberhard 1985). These spines, androgen-dependent structures (Arteaga-Silva *et al.* 2008), are found in bats, carnivores, primates and rodents. The presence and the morphology of the baculum and spines are highly variable between and within groups (Didier 1947; Burt 1960; Ryan 1991; Parag *et al.* 2006) and the divergence of these genital structures is extremely pronounced compared to the divergence of non-genital structures (Arnqvist 1998). It is now established that sexual selection (especially post-mating) is likely to have played a key-role in the evolution of genitalia in mammals and other animals (Short 1979; Eberhard 1985; Dixson & Anderson 2004; Hosken & Stockley 2004).

(b) Sexual selection and genital allometry

Hosken and Stockley (2004) reviewed four non-mutually exclusive sexual selection hypotheses proposed to explain genital evolution: sexual conflict, sperm competition, the Fisherian (or runaway) hypothesis (also called 'sexy sons' hypothesis) and the good genes hypothesis (**Figure 3.1**). The sexual conflict hypothesis postulates that male genitalia have evolved to increase male fitness even if there is some substantial cost for females (Crudgington & Siva-Jothy 2000; Arnqvist & Rowe 2005). Sperm competition, where sperm of two males are in competition to fertilize a set of ova (Parker 1970; Birkhead & Møller 1998; **Chapter 1**; **Chapter 4**), may have shaped genital morphology if some specific phenotypes confer an advantage to fertilize female eggs (Hosken & Stockley 2004; Hotzy & Arnqvist 2009). For instance, it has been suggested that male rodents could benefit from

longer genitalia due to a better ability to deposit copulatory plugs in a strategic position (Toner & Adler 1986) and/or sperm close to the site of fertilization (Ramm 2007). Other post-copulatory hypotheses invoke female cryptic choice to explain extreme divergence of male genitalia (Eberhard 1985; Hosken & Stockley 2004). Indeed, under a Fisherian process, females benefit from biasing paternity towards males who are the best stimulators since their sons will inherit this ability to stimulate females intensively (Eberhard 1985; Eberhard 1993; Hosken & Stockley 2004). The good genes hypothesis assumes also that males are chosen through the intensity of the stimulation provided, but in this case the progeny of these selected males show high quality (Hosken & Stockley 2004; Eberhard 2009). The good genes hypothesis is an extension of the Fisherian hypothesis but implies that genital size is condition-dependent (Hosken & Stockley 2004). Nevertheless, as emphasized by Eberhard (2001) there is no evidence that male genitalia can provide information on male parasite resistance or on male intrinsic quality (but see Arnqvist & Thornhill 1998). Moreover, the cost of producing these structures is probably low in species with relatively small and hidden genitalia (Eberhard 2009).

Counterbalancing these post-copulatory hypotheses, it has been suggested more recently that pre-copulatory sexual selection might play a role in genital evolution. Langerhans *et al.* (2005) showed in a live-bearing fish that males with larger gonapodia are more attractive to females, whereas Bertin and Fairbairn (2005) revealed in the water strider (*Aquarius remigis*) that larger genitalia are sexually selected in males because they confer to them a better ability to overcome female resistance during copulations (Fairbairn *et al.* 2003).

Current studies on genital evolution are now mainly focused on these sexual selection hypotheses, although initially non-sexual selection hypotheses were suggested (Roff *et al.* 2003; Hosken & Stockley 2004): the 'lock and key hypothesis' (Eberhard 1985) and the pleiotropy hypothesis (Mayr 1963). The 'lock and key hypothesis' assumes that genitalia evolved to favour hybridization avoidance. Hence males from a species are only able to copulate with females from the same species because male genitalia fit exclusively the genitalia of the conspecific females (Eberhard 1985; see also Shapiro & Porter 1989). The pleiotropy hypothesis considers that genitalia have evolved parallel to the evolution of other traits due to a pleiotropic effect of some genes (Mayr 1963). Evidence about the evolution of genitalia under non-sexually selective pressures remain scarce and it now appears more likely that sexual selection influences genital evolution, even if the relative importance of various

mechanisms described above needs to be determined (Hosken & Stockley 2004). Furthermore, hypotheses concerning the functions of the genitalia have been generally elaborated in a sexual selection context (Long & Frank 1968; Lariviere & Ferguson 2002; **Figure 3.1**).

(c) Functions of genitalia in mammals

If the obvious function of the male penis is to deposit sperm inside the female tract, the role of more elaborated genital traits such as baculum or spines is not so clear and several hypotheses have been proposed to explain the functions of these specific structures (Lariviere & Ferguson 2002; Stockley 2002). Generally, these functions are consistent with a role in post-copulatory sexual selection (sperm competition, female cryptic choice, sexual conflict; **Figure 3.1**). Long and Frank (1968) proposed that the role of the mammalian baculum might be to facilitate intromissions and to overcome vaginal friction during mating especially in species with strong sexual dimorphism (hereafter called the 'vaginal friction hypothesis') (Long & Frank 1968; see also Patterson & Thaeler 1982). The 'prolonged intromission hypothesis' suggests that an elongated baculum in species with prolonged intromissions may protect the urethra from compression during mating and consequently improve sperm transport (Dixson 1987; Verrell 1992; Dixson 1995). Another hypothesis is that the baculum might induce ovulation (Greenwald 1956). Therefore, individuals with an exaggerated baculum are more likely to cause ovulation and consequently to achieve successful fertilization. However, in a comparative study of North American carnivores, Lariviere and Ferguson (2002) failed to find evidence for any of these three hypotheses (i.e. 'vaginal friction', 'prolonged intromission' and 'induced ovulation'), since differences in baculum size were independent of sexual dimorphism, copulatory behaviour and ovulation type.

Similarly, functions of penile spines have been discussed in relation to post-copulatory sexual selection (Eberhard 1985; Harcourt & Gardiner 1994; Stockley 2002). For instance, penile spines could facilitate the displacement of previous copulatory plugs (Milligan 1979; Dunham & Rudolf 2009; but see Harcourt & Gardiner 1994) or stimulate the female reproductive tract (Zarrow & Clark 1968; Dewsbury 1972). This stimulation of the female reproductive tract might have two benefits for the males. It could improve sperm transfer via peristaltic contractions (Dewsbury 1972; but see Harcourt & Gardiner 1994) but also facilitate ovulation in species with induced ovulation (Zarrow & Clark 1968; Milligan 1982; Altuna & Lessa

1985; Harcourt & Gardiner 1994). Nonetheless, the first convincing function proposed for penile spines comes from Stockley (2002) who found in primates a significant negative relationship between the penile spinosity and the duration of female sexual receptivity (interestingly no relation is found between baculum length and sexual receptivity in this study) which suggests that penile spines might have evolved to reduce the risk of sperm competition through exaggerated female stimulation (Stockley 2002). Despite a lack of direct evidence in mammals, it is also possible that spines might damage the female reproductive tract, although this should be selected against due to possible consequences in terms of offspring survival (Stockley 2002).

(d) Allometry

Sexual selection is often predicted to result in positive allometry of the selected trait (Petrie 1992; Pomiankowski & Møller 1995; Kodric-Brown *et al.* 2006 and see **Figure 3.2**). A high phenotypic coefficient of variation of sexually selected traits (see below) is also expected to be associated with positive allometry (Petrie 1992; Pomiankowski & Møller 1995 but see Bonduriansky 2007; Eberhard *et al.* 2009; Eberhard 2009 and the discursive part of this chapter for the ongoing debate about sexual selection and gradient of the allometric slope). At this stage, it is important to specify that the use of the term 'allometry' in this thesis refers to 'static allometry' rather than 'ontogenic allometry'. That is, the following study aims to understand morphological variation between individuals of the same developmental stage ('static allometry') rather than variation between different developmental stages ('ontogenic allometry') (Bonduriansky 2007).

The allometric slope, generally called *b* (but sometimes also β), is calculated from the equation $Y = aX^b$ where Y is the dependent variable (e.g. genitalia length), X the independent variable (e.g. body length) and *a* the Y- intercept (Gould 1966; Sokal & Rholf 1995; Kodric-Brown *et al.* 2006). Thus, when both variables (dependent and independent) are log-transformed, the relationship becomes $\log(Y) = (b)\log(X) + \log(a)$. If the slope is not significantly different from 1, the trait investigated is isometric, which means that the size of the trait is proportional to body size. If the slope is significantly less than 1, the trait shows negative allometry, which means that smaller individuals will have a disproportionately larger version of this trait. If the slope is significantly greater than 1, larger males will have a disproportionately larger version of this trait (i.e. positive allometry; **Figure 3.2**) (Kodric-

Brown *et al.* 2006; Bonduriansky 2007; Eberhard 2009). Another parameter that has been used to investigate the type of selection acting on a trait is the coefficient of variation (CV), since it has been suggested that sexual selection is more likely to play a role in the evolution of a trait if the phenotypic variation of this trait is high (Pomiankowski & Møller 1995; House & Simmons 2003). However, Eberhard *et al.* (1998) emphasized that the coefficient of variation of a trait can be affected by the slope of the regression or the dispersion of the points around the regression line of this trait (**Figure 3.3**; see also Eberhard 2009). This observation leads Eberhard *et al.* (1998) to suggest another measure of phenotypic variation called CV' which is the coefficient of variation of the dependent variable if the independent variable is held constant (see **Methods**). This coefficient is calculated as: $CV' = CV * (1 - r^2)^{1/2}$ with r being the Pearson coefficient of correlation between the studied trait and body length. Despite the better accuracy of this coefficient in the assessment of phenotypic variation, its use remains scarce in the study of genital allometry (Eberhard 2009).

(e) Aim of the study

In species with dominance relationships, variation in male reproductive success is often linked to male social status (e.g. Widdig *et al.* 2004) but it is sometimes unclear why dominant males sire more offspring than subordinate males (e.g. Klemme *et al.* 2006a). Although several studies have compared the sperm allocation and/or ejaculate quality between dominant and subordinate males to understand the paternity bias toward high quality males (e.g. Rudolfsen *et al.* 2006; Pizzari *et al.* 2007; **Chapter 4**), relationships between genital morphology and male social status have not been investigated yet. The aim of the present study is to test in the bank vole (*Myodes glareolus*) for differences in genital traits[1] that might confer to dominant males an advantage in post-copulatory competition. This species is particularly suitable to test this since females mate multiply (Ratkiewicz & Borkowska 2000) and males have a social hierarchy with dominant and subordinate relationships (Viitala 1977). Dominant males have higher reproductive success than subordinate males (Klemme *et al.* 2006a; Kruczek & Zatorska 2008) even if, as pointed out by Klemme *et al.* (2006a), it is unclear if dominant males perform better in post-copulatory competition through an advantage in sperm competition, female cryptic choice or both. Male genitalia are complex in bank voles,

[1] In this chapter differences between dominant and subordinate males in genital morphology will be limited to the study of the baculum and penile spines. Differences in testis mass according to the male social status will be investigated in **Chapter 4**.

including a baculum and spines at the base of the penis (Milligan 1979). The baculum is constituted by a stalk, which is broad and elliptical in its proximal extremity and connected by cartilage to three ossified tiny bones in its distal part (Artimo 1964; **Figure 3.4**). The 'trident' shape of the bank vole baculum is found in some other rodents such as the red-backed vole (*Clethrionomys gapperi*) or the muskrat (*Ondathra zibethicus*) (Burt 1960; Tasikas *et al.* 2009). Females are induced ovulators and the stimulation provided by males during mating might influence their litter size (Klemme *et al.* 2007a).

Here I first tested if the structure of the baculum and penile spines differ in bank voles according to male social status. If it is the case, I predict that dominant males should have bigger genitalia which could explain their higher success in post-copulatory competition. Secondly I investigated if genital morphology is related to male copulatory behaviour and litter size in a non-competitive context. Finally I tested for evidence of positive allometry and a high coefficient of variation in the baculum and spines as generally predicted by models of sexual selection (Petrie 1992; Pomiankowski & Møller 1995; Kodric-Brown *et al.* 2006).

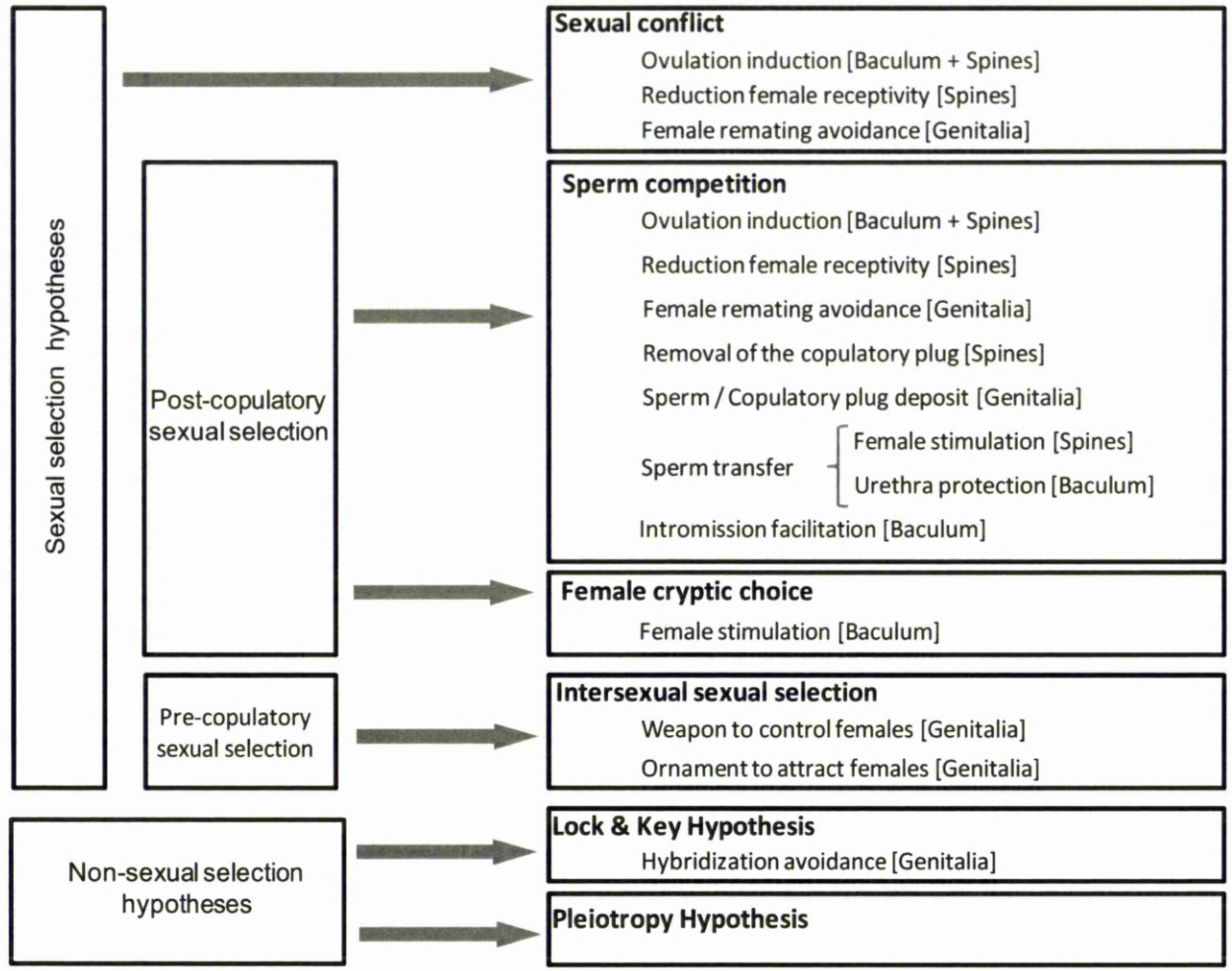

Figure 3.1 Summary of different selective pressures (left part) proposed to explain the evolution of the baculum and penile spines in mammals and associated possible functions (right part). On the diagram, [genitalia] means that the function can be attributed to bacula, spines or more generally to the penis.

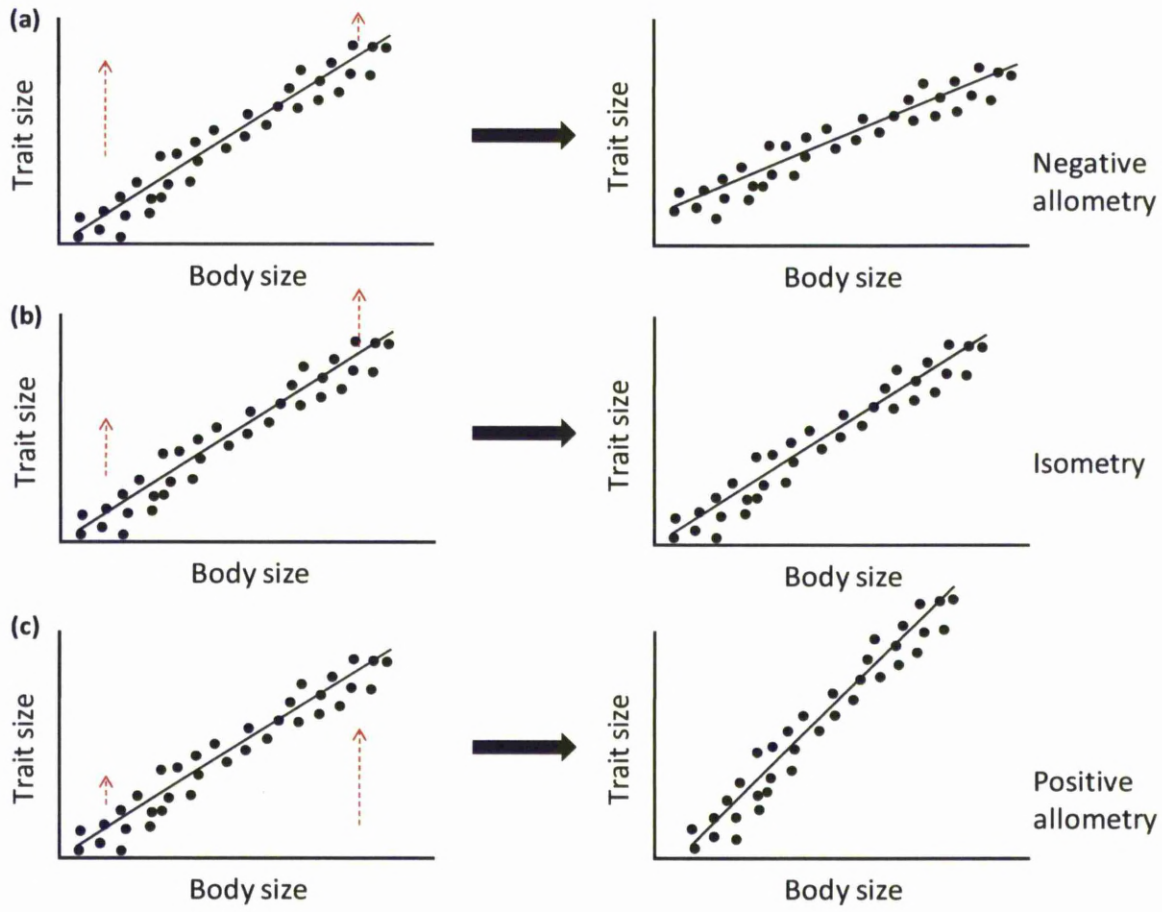

Figure 3.2 Effects of directional selection (after Eberhard *et al.* 2009). All slopes of the graphs from the left column show isometry ($\beta = 1$) but the directional selection on this trait will differ between individuals and will lead to different patterns of allometry. In the case (a), the intensity of selection is greater for small individuals who will have more benefits to increase the size of the trait compared to large individuals resulting in a negative allometry on the trait. In the case (c), the intensity of selection is greater for large individuals who will have more benefits to increase the size of the trait compared to small individuals resulting in a positive allometry whereas in the case (b) all individuals will have the same benefits to increase the size of the trait resulting in an isometric relationship.

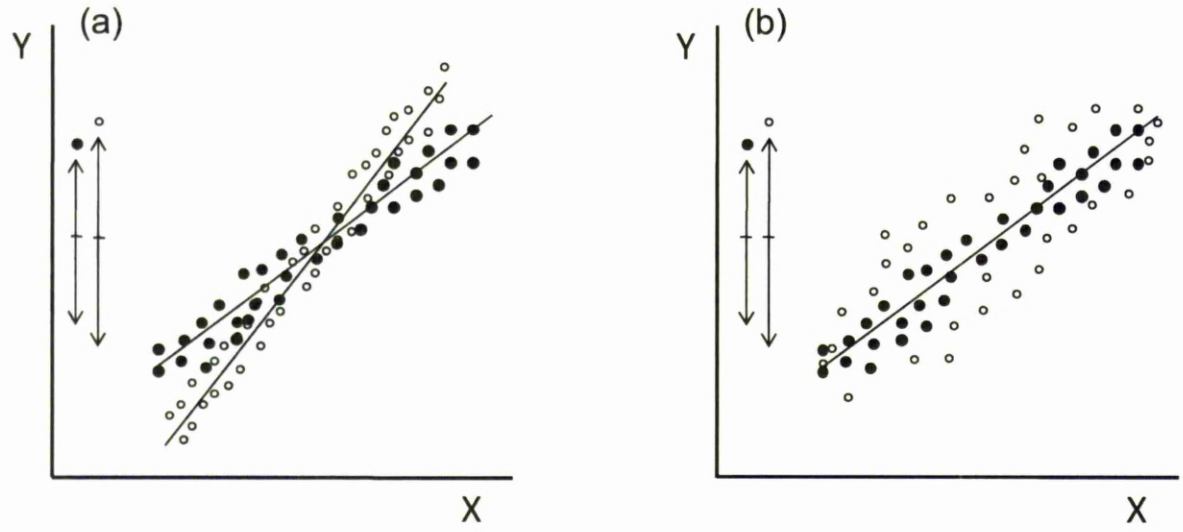

Figure 3.3 Statistical issues in the interpretation of the coefficient of variation (CV) (after Eberhard *et al.* 1998). The coefficient of variation is not independent of the slope of the regression and of the distribution of the points around the allometric line. In the case (a), open circles and solid circles have the same mean but because the slope of the regression line is steeper for the open circles, the standard deviation (arrows) of this trait is higher. In the case (b), the greater standard deviation for the open circles has for origin the greater dispersion of points around the regression line. In both cases the increase of the standard deviation will lead to an increase of the coefficient of variation.

3.3 Methods

(a) Subjects

Bank voles used in the experiments were the adult F1 and F2 offspring of 29 wild-caught individuals (15 males and 14 females) trapped in Cheshire (UK) between January and May 2007 (see also **Chapter 2**, **4** and **5** of the thesis for a description of the colony). After weaning, males were housed with their brothers in MB1 cages (40 X 23.5 X 20 cm, North Kent Plastic Cages Ltd., UK) containing substrate (Corn cob Absorb 10/14 substrate) and paper-wool bedding material (hereafter called bedding). Food and water were provided *ad libidum* (LabDiet 5002). Animals were maintained on a reversed photoperiod (light: 16hrs, dark: 8hrs, lights on at 17h00), and at a temperature of 21 ± 1°C. Genitalia analysed in this

study come from two groups of adult males, previously used for different experiments (**Chapter 4**).

Briefly, males from 'Group 1' (n = 27) were used for an experiment designed to understand differences in male copulatory behaviour between dominant and subordinate males, and males from 'Group 2' (n = 21) were used in an experiment designed to assess differences in sperm allocation between dominant and subordinate males (see **Chapter 4** for the complete details of the experimental design). All these males were placed with an unrelated female in an arena and had the opportunity to mate at least until the first ejaculation (see **Chapter 4** for the complete details of this experimental design).

To compare the coefficient of phenotypic variation of the baculum and spines with a trait less likely to be influenced by sexual selection, I choose the third metatarsal bone of the right hind foot (see **Section 3.3.e**). Males used to measure the metatarsal bone come from an experiment designed to assess the sperm production of male bank voles in relation to the level of exposure to social odours (see **Chapter 5** for complete details of the experimental design). The social status of these males is unknown.

(b) Social dominance establishment

Male subjects were housed in MB1 cages divided in two by a mesh barrier, with one male of each pair housed in each of the two parts of the cage (Each part: 20 X 11.75 X 20 cm) over a period of 10 to 30 days. Therefore the two males paired were in continuous olfactory and visual contact until the end of the experiment. To identify unambiguously dominant-subordinate pairs, I observed male urinary marking behaviour, which is a technique commonly used to determine dominance relationships in rodents and particularly in bank voles (Rozenfeld *et al.* 1987; Horne & Ylönen 1996; Klemme *et al.* 2006a). To assess scent marking behaviour, both males from a pair were transferred to clean benchkote-lined MB1 cages (again divided in two by a mesh barrier) and left for 30 min (in the dark phase period) without external stimulus added. Scent marks were scanned using Bio-rad Fluor-S^{TM} MultiImager (QuantityOne software: 12sec exposure, 530DF60 Filter, UV light source Epi illumination, high resolution mode) and analyzed using Scion image software. The social status of the males was assessed based on the criteria identified by Rozenfeld and Rasmont (1991), with thin streaks of urine throughout the arena characteristic of dominant males and

large puddles of urine, especially in the corner of the arena, typical for subordinates. Only pairs with an unambiguous dominant-subordinate relationship were used in our analyses (see also **Chapter 2** for details concerning social dominance establishment).

Social dominance establishment was strictly the same between males from 'Group 1' and males from 'Group 2'. Therefore, I pooled data for males from both these groups in order to perform comparisons of male genitalia according to social status.

(c) Mating trials

Each male was placed with an unrelated female in a neutral arena (70 x 60 x 50 cm). Males were placed first in the arena. I next added the female to the arena using a Perspex tube (diameter = 5 cm) closed at one end by a mesh barrier. I put the open end of the tube against one of the wall of the arena for 10 minutes in order to allow individuals to acclimate to each other through only visual and olfactory contact. The tube was then removed and females were in direct contact with males. If nothing occurred (mounts or intromissions) during the first 30 min of the experiment or if persistent aggression occurred between the two individuals, the female was removed and replaced by a new unrelated female (see Klemme *et al.* 2006a). Matings were stopped after the male's last ejaculation (if nothing occurred in the next 30 min following an ejaculation it was considered that the male had mated to satiety) for males of the 'Group 1' or after the first ejaculation for males of the 'Group 2'. All copulations were recorded on a DVD using a CCTV video stream relayed to an adjoining room allowing rapid interventions in case of aggressive behaviour. All measured copulatory behaviours are described in **Section 3.3.d** and **Chapter 4**). Males were euthanized and dissected 1 week after completion of the mating.

(d) Copulatory behaviour and litter size

DVDs were analysed and male and female behaviour was quantified. The bank vole copulatory behaviour is generally described as several ejaculation series constituted by mounts (without vaginal penetration), intromissions (vaginal penetration without sperm transfer) and ejaculation (Milligan 1979; Klemme *et al.* 2006a). During the ejaculation, males generally start to quiver and then fall to one side taking away the female with them (Milligan 1979). Since one aim of this study was to investigate the relationship between the male genital

morphology and the level of stimulation provided by males to females, I recorded three different characteristics of the copulatory sequence of each male: the number of intromissions, the intromission rate (number of intromissions per minute between the first intromission and the first ejaculation) and the ejaculation latency (time from the first intromission to the first ejaculation) (see also **Chapter 4**). Twenty-five of 27 males mated to satiety with a female. These females were followed until they gave birth and the number of pups produced following copulations with each male was counted.

(e) Preparation and measurement of bacula, spines and metatarsal bones

All individuals were euthanized using halothane and were killed by dislocating the neck. Measures of body length (to the nearest 0.01 mm) were taken just after death using a digital calliper.

The penis of each male was removed and frozen prior to further analysis. Next, penises were defrosted at room temperature and the majority of the tissue around the baculum was removed with forceps under a microscope with 20X objective. Then, following the method commonly used (Friley 1947; Kinahan *et al.* 2007; Tasikas *et al.* 2009; Ramm *et al.* 2010), a series of dissections was combined with soaking for one to two hours in 1ml of KOH at a concentration of 0.05 gml^{-1}. When the baculum was perfectly cleaned of surrounding tissue, the bone was stored in 1ml of 70% ethanol solution. The procedure was exactly the same for removal and cleaning of the third metatarsal bone of the right hind foot.

Since the morphology of the bank vole baculum is very similar to the baculum shape of the muskrat, I followed the same procedure as Tasikas *et al.* (2009) in their analysis of this rodent baculum. Consequently, four measurements (to the nearest 0.001 mm) were recorded on the baculum (**Figure 3.4**): the total length (TL), the central length (CL), the central width (CW) and the base width (BW). Similar to Ramm *et al.* (2010), I measured the length of the metatarsal bone (MtL) and I added a measure of the width of this bone (MtW) (both measures to the nearest 0.001mm).

Morphological measurements taken from the baculum and the metatarsal bone were obtained using a flatbed scanner (CanoScanLiDE 30, Canon Inc.) at a resolution of 1200 dpi to obtain a highly contrasted image. The method to analyse the scan was similar to the method used by

Ramm *et al.* (2010). Scans were imported into ImageJ software (version 1.38x, http://rsbweb.nih.gov/ij/), inverted and converted to 32-bit. To facilitate the measures, I rotated the pictures to align the baculum shaft on a vertical axis. Measures were taken only when bones were sufficiently intact to perform accurate analyses, which explain the difference in sample size between each measure. Two persons performed the measures and their results were highly repeatable (e.g. for baculum total length: intra-class correlation coefficient: $r = 0.83$; $F_{19} = 12.49$; $P < 0.001$).

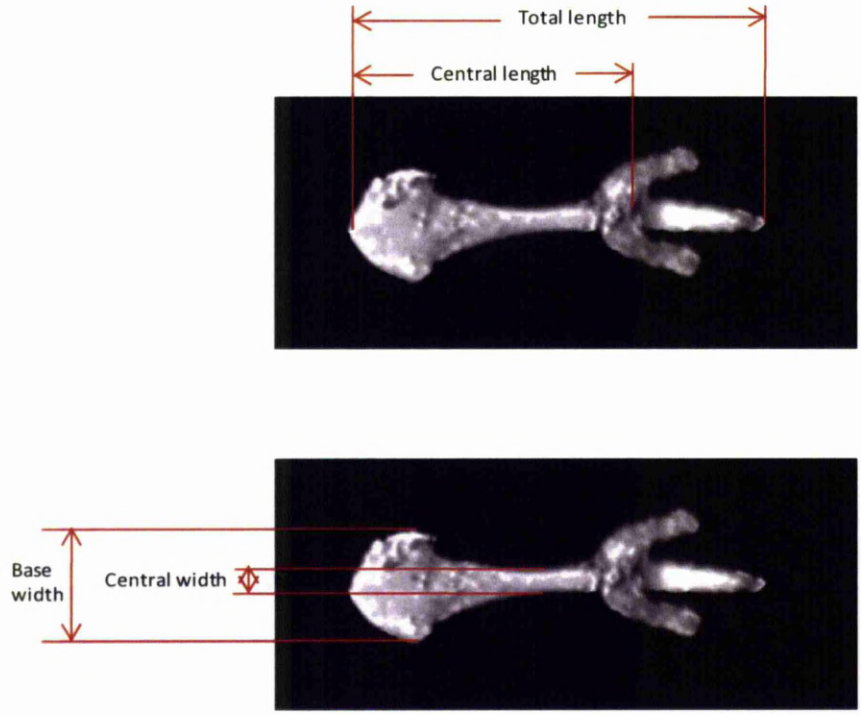

Figure 3.4 Measures of the bank vole baculum. I followed the same method as Tasikas *et al.* (2009) who studied the closely similar baculum of the muskrat and measured four traits: the total length (TL), the central length (CL), the central width (CW) and the base width (BW).

(f) Preparation and measurement of penile spines

Penises were removed from the fridge and transferred to the dissection place in dry ice. They were first placed on frozen fixative (2.5% gutaraldehyde, 4% formaldehyde in 100mM sodium phosphate buffer) to thaw at room temperature. After a night of fixing at room temperature on a rotator, samples were washed twice (40 min each time) with a solution of 100mM sodium phosphate buffer. Next, samples were submitted to a series of dehydrations

(45 min each) with ethanol of increasing concentration (25%, 50%, 70%, 90% and 2 X 100%) and were treated with hexamethyldisilazane (HMDS) for 1 hour. Hereafter, tops were removed from vials and most of the HMDS as well. The remainder was allowed to evaporate off in a desiccator overnight. Penises were mounted using silver-dag (conductive silver paste) on standard aluminium stubs for scanning electron microscopy and sputter coated with gold/palladium. Finally specimens were viewed using a scanning electron microscope JSM 6490LV (JEOL, Tokyo, Japan) running at 5kv and images were taken at low magnification (**Figure 3.5**). To determine the different information needed, images were taken x30 to see the overall specimen, x60 to show the whole band of spines, x200 in the centre of the band and x200 out of the centre of the band.

Spines were analysed using ImageJ software (version 1.38x, http://rsbweb.nih.gov/ij/). The x30 pictures were used to assess the surface covered by the spines on the penis (**Figure 3.5**). To determine the length of the spines, 15 spines on the x200 centre pictures were measured and the mean of these values was calculated. The size was measured from the tip to the base of the spines (to the nearest 0.001 mm) (**Figure 3.6**). Finally, to determine spine density all spines were counted (using the cellcount Plugin for ImageJ) on the x200 centre pictures and divided by the surface they covered.

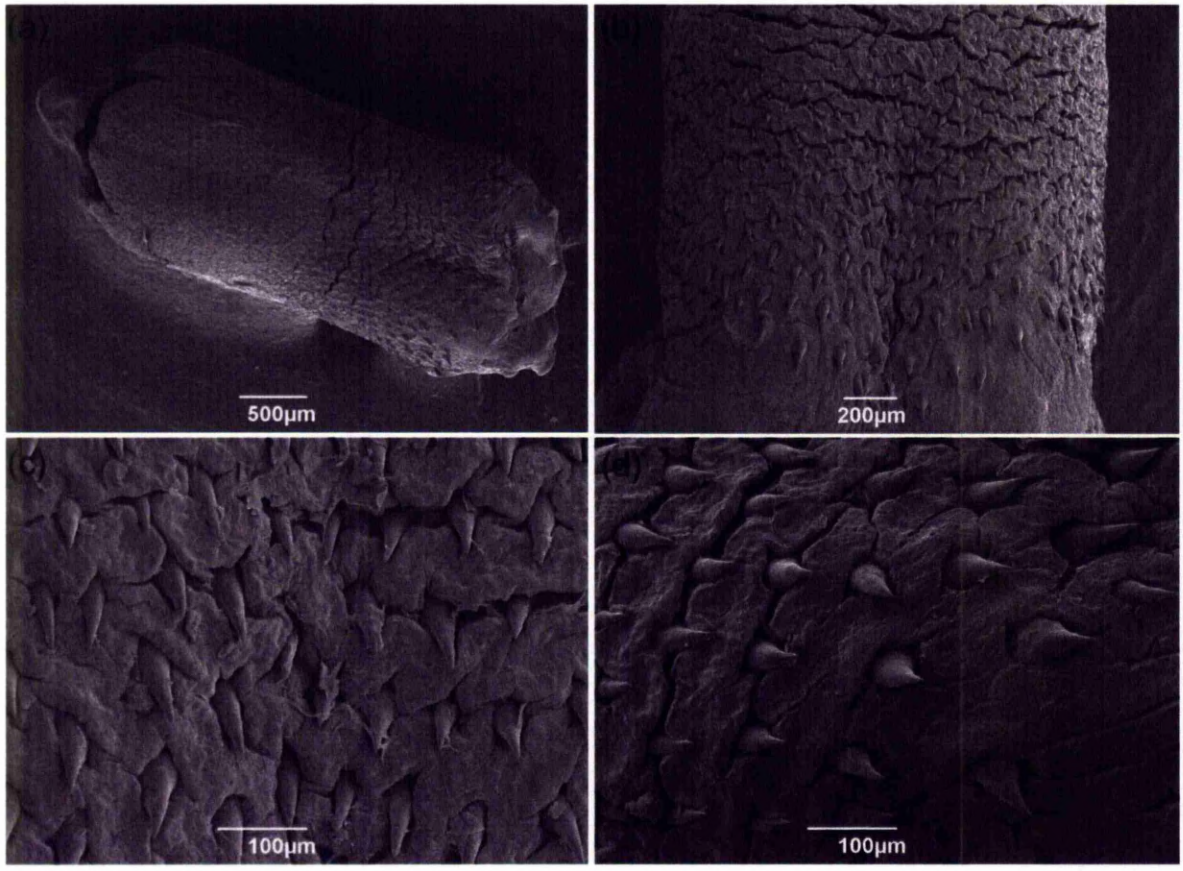

Figure 3.5 Scanning electron microscope images of the adult bank vole penis. On (a) we can see the whole penis whereas on (b) it is limited to the surface of the penis covered by spines. These two pictures show that spines are found near the proximal end of the penis. On (c) and (d), we can see more clearly the morphology of the penile spines. The magnification of (a) is x30, of (b) x60 and of (c) and (d) x200.

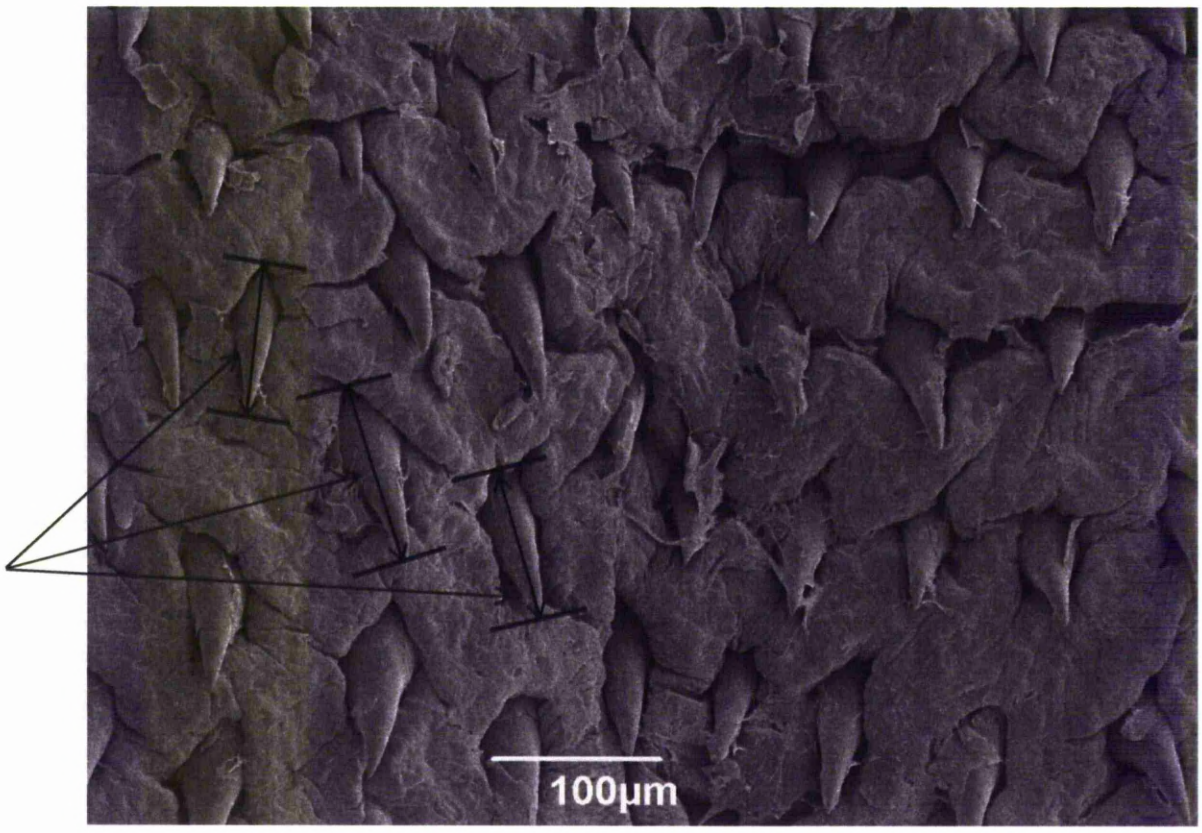

Figure 3.6 Measures of penile spine length. The arrows show the measure taken on the spine, from the tip to the middle of the base of this structure.

(g) Statistical analysis

Before performing the analysis to explain litter size by copulatory behaviour and genital morphology, I first performed a principal component analysis (PCA) to reduce the number of predictors. The PCA focused on the four baculum log-transformed dimensions (TL, CL, CW and BW). The first eigenvector factor extracted from the PCA summarized multivariate baculum dimensions explained 51.69% of the variance and had an eigenvalue of 2.07. The loadings of the four baculum measurements on this first factor were: 0.92 (TL); 0.79 (CL); 0.15 (CW); 0.75 (BW). The factor score (FS baculum morphology) is thus the single variable used in the subsequent analysis to represent baculum dimensions (hereafter called 'baculum morphology factor'). Then I performed a second PCA on the three spines dimensions (spine length, density and coverage). The first eigenvector factor extracted from the PCA summarized spines dimensions explained 71.90% of the variance and had an eigenvalue of

2.16. The loadings of the three spines measurements on this first factor were: 0.81 (spines length); 0.85 (spines density); 0.88 (spines coverage). The factor score (FS spines morphology) is thus the single variable used in the subsequent analysis to represent the spines dimensions (hereafter called 'penile spinosity factor').

The baculum and control traits were analyzed for positive allometry by performing ordinary least squares (OLS) regression on log-transformed data. OLS regression is adapted to test if the slope of the regression line significantly deviates from zero (ß = 0). Transformed data of each trait were entered in the model as the dependent variable and transformed data of body length were entered in the model as the independent variable. However, there are some potential issues in the use of OLS regression in tests of allometric relationships. The model assumes that Y and X are not interdependent and the absence of error in X (Sokal & Rohlf 1995). Therefore, I followed advice from previous studies (Tasikas *et al.* 2009; Lüpold *et al.* 2004) and used the reduced major axis regression (RMA) (model II) to estimate the slope value of the regression line of the various traits tested. Finally, t-tests were used to test if the estimated slopes were significantly different from isometry (ß = 1) (see Eberhard *et al.* 1998; Lüpold *et al.* 2004).

To compare phenotypic variation between putative sexually selected traits, the coefficient of variation (CV) as (s.d/mean)*100 was calculated. Because some statistical issues can affect the interpretations of CV values (see **Figure 3.3**), advice from Eberhard *et al.* (1998) was followed and CV' as $CV*(1-r^2)^{1/2}$ was calculated with r being the Pearson coefficient of correlation between the studied trait and body length. CV' is the coefficient of variation of the investigated trait if body size was held constant (Eberhard *et al.* 1998). As emphasized by Eberhard *et al.* (2009), it is important to be cautious in the interpretation of the coefficient of variation and it should not be interpreted alone but in comparison with the coefficient of variation of other traits.

All other statistical tests were conducted on log-transformed data using SPSS 16.0. Data are presented as means ± s.e.m (except for the coefficient of variation presented as means ± s.d) and differences were regarded as statistically significant at $P < 0.05$.

3.4 Results

(a) Genital morphology, litter size and male social status

Dominant and subordinate males did not differ in age (dominants: $\bar{x}$ = 250.04 ± 13.71 days; subordinates: $\bar{x}$ = 233.96 ± 17.34 days; t_{46} = 0.72, P = 0.47) or in body weight (dominants: $\bar{x}$ = 26.53 ± 4.62 g; subordinates: $\bar{x}$ = 24.83 ± 3.90 days; t_{46} = 1.27, P = 0.21). Dominant males had significantly larger basal width of the baculum than subordinate males (dominants: $\bar{x}$ = 1.34 ± 0.19 mm; subordinates: $\bar{x}$ = 1.18 ± 0.15 mm; t_{27} = 2.41, P = 0.02) but the baculum total length, baculum central length, baculum central width, spines length, spines coverage and spines density did not differ according to male social status (**Table 3.1**). No significant difference in litter size was found between dominant and subordinate males (dominants: $\bar{x}$ = 3.00 ± 2.15; subordinates: $\bar{x}$ = 2.18 ± 2.14; n = 25; Z = -0.91, P = 0.40) and none of the genital traits predicted the litter size (**Table 3.2**). When the 9 males (4 dominants and 5 subordinates) who failed to sire offspring (i.e. litter size = 0 pup) were removed from the analysis, all results were qualitatively unchanged. Indeed, no significant difference in litter size was also found (dominants: $\bar{x}$ = 4.20 ± 0.33; subordinates: $\bar{x}$ = 4.00 ± 0.26; n = 16; Z = -0.34, P =0.79) and none of the genital traits predicted the litter size (not shown) but the very low sample sizes used in these analysis do not allow reliable interpretations ($5 \leq n \leq 7$).

Table 3.1 Differences between dominant and subordinate males in all genital traits investigated. Only the basal width of the baculum differs significantly in relation to male social status.

Trait	Social status	*n*	Mean	s.e.m	*t*	*P*
Baculum total length (mm)	Dominant	10	4.04	0.06	1.17	0.25
	Subordinate	11	3.92	0.09		
Baculum central length (mm)	Dominant	14	2.71	0.07	-0.08	0.94
	Subordinate	12	2.71	0.06		
Baculum central width (mm)	Dominant	16	0.34	0.02	1.90	0.07
	Subordinate	17	0.30	0.01		
Baculum basal width (mm)	Dominant	14	1.34	0.05	2.40	0.02
	Subordinate	15	1.18	0.04		
Spines length (μm)	Dominant	10	70.59	3.67	-0.27	0.79
	Subordinate	10	72.35	5.43		
Spines coverage (%)	Dominant	10	32.01	1.64	0.59	0.56
	Subordinate	10	29.92	3.14		
Spines density (sp/μm²)	Dominant	10	103.32	5.57	0.76	0.45
	Subordinate	10	95.69	8.31		

(b) Genital morphology, copulatory behaviour and male social status

I found no relationship between any of the copulatory traits investigated (number of intromissions, intromission rate and ejaculation latency) and the structure of the genitalia (**Table 3.3**) except that the number of intromissions positively correlated with the total length of the baculum (TL) (n = 20, r = 0.458, P = 0.042) and negatively correlated with the length of the penile spines (n = 20, r = -0.449, P = 0.047). As pointed out above, I found no correlation between each genital trait and the litter size (**Table 3.2**). However, since the litter size might be explained by the copulatory behaviour only or by the interaction between the morphology of the genitalia and the copulatory behaviour, I ran general linear models (GLM) including litter size as the dependent variable and two independent variables: a copulatory trait involved in female stimulation (number of intromission or intromission rate) (see Methods) and a genital trait ('baculum morphology factor', 'penile spinosity factor' or baculum basal width). Indeed, because the baculum basal width was the only dimension significantly different between dominant and subordinate males, I tested it solely. Therefore six models were performed to explain litter size but none revealed evidence of a significant

effect of the genital trait, copulatory behaviour trait or the interaction between the genital trait and the copulatory behaviour trait (results not shown).

Table 3.2 Correlation between litter size and genital traits. For the baculum total length, baculum central length, baculum central width and the baculum basal width the coefficient of Pearson correlation is calculated between the litter size ($0 \leq n \leq 6$) and the log-transformed value of the trait. These results are qualitatively unchanged when males who failed to sire offspring were removed from the analysis (see text).

Trait	*n*	*r*	*P*
Baculum total length	11	0.37	0.26
Baculum central length	13	0.39	0.19
Baculum central width	17	-0.06	0.83
Baculum basal width	14	-0.22	0.44
Penile spines length	14	0.25	0.38
Penile spines density	14	0.06	0.85
Penile surface covered by spines	14	0.35	0.22

Table 3.3 Pearson coefficient of correlation between each genital and copulatory trait investigated. The number of intromissions positively correlated with the total length of the baculum (TL) (n = 20, r = 0.46, P = 0.042) and negatively correlated with the length of the penile spines (n = 20, r = -0.45, P = 0.047).

	n	Number of intromissions	Intromission rate	Ejaculation latency
Baculum total length	20	0.46*	0.39	-0.08
Baculum central length	24	0.39	0.17	0.13
Baculum central width	29	0.34	0.21	0.04
Baculum basal width	26	0.22	-0.09	0.27
Penile spines length	20	-0.45*	-0.15	-0.21
Penile spines density	20	-0.13	0.18	-0.36
Penile surface covered by spines	20	-0.36	-0.14	-0.14

(c) Allometry and phenotypic variation

Slopes for baculum central length (CL) and baculum basal width (BW) were significantly greater than 0 with ordinary least squares regressions (OLS) (**Table 3.4**). Nevertheless, using the reduced major axis regression (RMA) only the allometric slope of basal width was significantly greater than 1 ($ß_{RMA}$ = 2.52) which is similar to the results found by Tasikas *et al.* (2009) for the muskrat baculum and to some extent to the results found by Ramm *et al.* (2010) for the house mouse baculum. Finally, I found no evidence of positive allometry for penile spines length and none of the allometric slopes for the metatarsal traits was significantly greater than 0 (**Table 3.4**).

As emphasized by Eberhard *et al.* (2009), coefficients of variation have to be interpreted in comparison to each other. In my dataset, four dimensions have a coefficient of variation (CV') greater than 10%: the baculum central width (16.98 %), the baculum basal width (14.92 %), the spines length (20.00 %) and the metatarsal width (10.79 %) (**Table 3.4**). A coefficient of variation above 10 % is relatively high and more likely to be found for traits influenced by sexual selection (Lüpold *et al.* 2004; Manjerovic *et al.* 2008; Tasikas *et al.* 2009; Ramm *et al.* 2010).

Table 3.4 Phenotypic variation and statistics for ordinary least square (OLS) and reduced major axis (RMA) regressions. Slopes are calculated for the four baculum measurements, the length of the penile spines and the two metatarsal measurements. RMA slopes are calculated only when the OLS slope is significantly different from zero. The variation of each trait is assessed by the coefficients of variation: CV and CV' (see Method for a description of the calculation). $*P < 0.05$; $**P < 0.01$

			Phenotypic variation				OLS (slope H0 = 0)				RMA (slope H0= 1)		
Trait (mm)	Individuals	N	Mean	s.d.	CV (%)	CV' (%)	*r*	Slope	s.e	t	Slope	s.e	t
Baculum Total Length	All	21	3.98	0.25	6.33	6.16	0.23	0.26	0.252	1.03	-	-	-
	Dominants	10	-	-	-	-	0.40	0.35	0.285	1.22	-	-	-
	Subordinates	11	-	-	-	-	0.03	0.04	0.449	0.08	-	-	-
Baculum Central Length	All	26	2.71	0.23	8.66	7.80	0.44	0.64	0.272	2.37*	1.48	0.27	1.75
	Dominants	14	-	-	-	-	0.48	0.73	0.389	1.87	-	-	-
	Subordinates	12	-	-	-	-	0.42	0.64	0.435	1.46	-	-	-
Baculum Central Width	All	33	0.32	0.05	16.98	16.35	0.26	0.76	0.498	1.52	-	-	-
	Dominants	16	-	-	-	-	0.31	0.80	0.649	1.24	-	-	-
	Subordinates	17	-	-	-	-	0.17	0.49	0.742	0.65	-	-	-
Baculum Basal Width	All	29	1.26	0.19	14.92	13.47	0.42	1.05	0.441	2.38*	2.52	0.44	3.44**
	Dominants	14	-	-	-	-	0.63	1.47	0.53	2.78*	2.36	0.53	2.56*
	Subordinates	15	-	-	-	-	0.10	0.22	0.64	0.35	-	-	-
Spines length	All	20	0.07	0.01	20.00	19.96	0.06	-0.35	1.388	-0.25	-	-	-
	Dominants	10	-	-	-	-	0.45	-1.80	1.261	-1.43	-	-	-
	Subordinates	10	-	-	-	-	0.19	1.43	2.643	0.54	-	-	-
Metatarsal length		27	7.24	0.17	2.29	2.29	0.005	-0.003	0.114	-0.03	-	-	-
Metatarsal Width		27	0.56	0.06	10.79	10.63	0.17	0.47	0.536	0.88	-	-	-

3.5 Discussion

(a) Male social status and genitalia morphology

In bank voles, dominant males generally sire more offspring than subordinate males (Klemme *et al.* 2006a; Kruczek & Zatorska 2008) through an advantage in sperm competition and/or female cryptic choice (Klemme *et al.* 2006a). Indeed, in species where females mate multiply such as bank voles (Ratkiewicz & Borbowska 2000; Klemme *et al.* 2007a), if the investment in genitalia is an honest signal of male quality, females might use the size of this structure to select males (Eberhard 1985; Hosken & Stockley 2004). My results are in accordance with this hypothesis since the basal part of the baculum is larger for dominant males than subordinates and consequently may provide more stimulation to females (Eberhard 1985; Hosken & Stockley 2004). Ramm (2007) pointed out that in rodents baculum length and sperm competition level are positively correlated, and suggested that an elongated baculum could help in the deposition of the ejaculate close to the site of fertilization. However, in bank voles the advantage of dominant males in post-copulatory competition cannot be explained by an elongated baculum since the length (total or central) of this bone does not differ between dominant and subordinate males. Therefore, if dominant males perform better in sperm competition than subordinate males, it might come from differences in sperm and/or seminal fluid allocation (see Wedell *et al.* 2002; Kruczek & Styrna 2009; **Chapter 4**) rather than differences in genital morphology (i.e. baculum length and penile spines).

Contrary to my predictions, the degree of spinosity did not differ in relation to male social status and therefore cannot explain the higher reproductive success of dominant males. Evidence so far tends to support the hypothesis that penile spines evolved under sperm competition and/or sexual conflict pressures (Hosken & Stockley 2004; Dunham & Rudolf 2009) such as in primates where penile spinosity is negatively associated with the duration of female receptivity and consequently may reduce sperm competition risk (Stockley 2002). The evolution of penile spines under female cryptic choice seems unlikely due to the risk of injuries caused by this sharp structure (Stockley 2002) and logically has received less support (Harcourt & Gardiner 1994). Further experiments are required to understand the evolutionary significance of penile spinosity in rodents especially in induced ovulator species such as bank voles, since it has been proposed that female stimulation through spines could initiate a cascade of reactions leading to the ovulation (Milligan 1979; see also below).

Nonetheless, no relation was found here between genital morphology and litter size, at least under non-competitive conditions. However, to test for an advantage provided by genitalia, it will be important to also compare the reproductive success of males who have mated with the same female (e.g. Arnqvist & Danielsson 1999). There is no evidence yet that the morphology of male's genitalia in mammals is related to fertilization success, whereas this is the case in several invertebrate species (House & Simmons 2002; see also Hosken & Stockley 2004 for a review). For example, in two species of water strider (*Gerris lateralis* and *Gerris Lacustris*) there is a correlation between the male genital morphology and fertilization success (Arnqvist & Danielsson 1999; Danielsson & Askenmo 1999).

Several hypotheses, rarely tested in mammals, have suggested that genitalia could improve male fitness by playing a role during copulation (Milligan 1979; Dixson 1987). In a comparative study across rodents, Ramm (2007) did not find a relationship between the length of the baculum and the number of intromissions. However, I found here that in bank voles the length of the baculum appears to be positively correlated with the number of intromissions, which corroborates the idea that the baculum might facilitate intromissions (Long & Frank 1968; Patterson & Thaeler 1982). This result needs to be interpreted cautiously, especially since the increased number of intromissions associated with baculum morphology does not provide benefits of increased litter size. More recently, it has been argued that an elongated baculum might help in sperm transport through protection of the urethra in species with prolonged intromissions (Dixson 1987). This hypothesis has received mixed support (e.g. Dixson 1995; Lariviere & Ferguson 2002) and applies to species with prolonged intromissions which is not the case in bank voles that typically perform multiple short intromissions (Milligan 1979; Pers. obs.). Intromissions have an important role of female and male stimulation in rodents (Dewsbury 1982b) and penile spines might enhance this stimulation which could allow males to decrease the overall number of intromissions performed (Milligan 1979; see also Harcourt & Gardiner 1994). My findings tend to support this idea since the length of the spines is negatively correlated with the number of intromissions. Therefore, in bank voles, elongated spines might decrease the minimum level of stimulation required prior to ejaculation. Similarly, Dixson (1991) found that male marmosets (*Callithrix jacchus*) with penile spines experimentally removed increase the duration of pelvic thrusting before ejaculation (Dixson 1991), suggesting also that penile spines probably improve male tactile sensitivity during copulation (see also Beach & Levinson 1950). However I did not find a correlation between the length of the penile spines

and ejaculation latency, suggesting that a decrease in the number of intromissions does not automatically reduce the duration of copulation.

(b) Allometry and phenotypic variation in bank voles

Similar to the pattern found in some other rodents (Ramm *et al.* 2010; Tasikas *et al.* 2009) or carnivores (Miller & Nagorsen 2008), the baculum basal width of bank voles displays a positive static allometry. Direct comparisons with some other species remain difficult since the width of the baculum (as opposed to its length) has only rarely been included in previous analyses (e.g. Miller *et al.* 1999; Lüpold *et al.* 2004; Kinahan *et al.* 2007). Contrary to the results reported here, baculum length generally exhibits a positive allometry in mammals (Miller & Burton 2001; Kinahan *et al.* 2007; Tasikas *et al.* 2009). Nevertheless, this relationship is not universal, for instance in the house mouse (*Mus musculus*) the scaling relationship of baculum length is isometric (Ramm *et al.* 2010; but see also Miller *et al.* 1999; Miller *et al.* 2000). I found that the length of bank vole penile spines does not exhibit positive allometry but direct comparisons with other mammalian species are difficult due to the absence of information in the literature.

The coefficient of phenotypic variation of baculum width (central and basal) and spine length are higher compared to metatarsal traits (assumed to be non-sexually selected), as previously found for other mammals (Kinahan *et al.* 2007; Manjerovic *et al.* 2008; Tasikas *et al.* 2009 but see Dyck *et al.* 2004). This high coefficient of variation associated with a positive allometry suggests that the baculum basal width is likely to be influenced through directional sexual selection (Petrie 1992; Pomiankowski & Møller 1995; Kodric-Brown *et al.* 2006). However, the absence of positive allometry found for penile spines length does not necessarily mean that sexual selection does not play a role in the evolution of this structure (Bonduriansky & Day 2003; Bonduriansky 2007). Indeed, interpreting genital allometry is generally not simple in regards to the different structures involved. For example, in bats, the penis length rather than the baculum exhibits positive allometry (Lüpold *et al.* 2004) whereas in the African mole-rat, both penis and baculum show positive allometry (Kinahan *et al.* 2007). Ultimately, establishing the nature of selection acting on genital traits requires informations on the benefits conferred by these traits in term of reproductive success (e.g. House & Simmons 2003).

Evidence of positive allometry in mammalian genitalia (e.g Miller & Burton 2001; Kinahan *et al.* 2007; Tasikas *et al.* 2009) contrasts with the pattern of negative allometry generally found in insects (Eberhard *et al.* 1998). Nevertheless, this negative relationship is not incompatible with the evolution of genitalia under sexual selection (Eberhard *et al.* 1998; Bonduriansky & Day 2003) due to combined implication of stabilizing and directional selection (Eberhard *et al.* 2009). Indeed the most likely explanation, named the 'one size fits all' hypothesis (Eberhard *et al.* 1998), is that intermediate size for genitalia are favoured since it allows males to stimulate a large range of females (Eberhard *et al.* 1998). Furthermore, there are ongoing discussions on the link between sexual selection on a trait and the pattern of allometry exhibited by this trait (Bonduriansky 2007; Eberhard 2009). In a wide context including sexual selection on weapons and ornaments, Kodric-Brown *et al.* (2006) predict that positive allometry of a trait will occur if this trait is subject to sexual selection, whereas Bonduriansky and Day (2003) predict positive allometry on sexually selected traits only if some conditions are respected. These limited conditions formulated by Bonduriansky and Day (2003) contrast with the long list of sexually selected traits showing positive allometry provided by Kodric-Brown *et al.* (2006) to support the output of their model. Bonduriansky (2007) emphasized that a sampling bias for exaggerated traits in morphometric studies (e.g. fiddler crab claws) has lead to an overestimation of the proportion of sexually selected traits exhibiting positive allometry. Focusing on genitalia, Eberhard (2009) corroborates Bonduriansky's (2007) conclusions by compelling results from a large range of studies and reveals mainly in arthropods various cases of negative allometry in genital structures. Bonduriansky and Day (2003) argue that a sexually selected trait can show a large variety of allometric patterns (see also van Lieshout & Elgar 2009). Results here for penile spines tend to confirm these predictions since due to their position on the body, it is likely that at some point in the evolutionary history the sexual selection process has played a role on the evolution of this structure. Unfortunately, the scaling relationship of penile spines is rarely described in mammals and future studies should investigate this to see if the absence of pattern found in bank voles is the rule for this structure or a particular case due to some specific aspect of the species' ecology or life history.

3.6 Conclusion

Bank vole genitalia are complex, and include both a baculum and penile spines. Partly consistent with some other mammals (Miller & Nagorsen 2008; Ramm *et al.* 2010; Tasikas *et*

al. 2009), the bank vole baculum basal width shows a positive allometry. This positive allometry associated with a high phenotypic variation suggests that this structure is likely to be influenced by sexual selection (Pomiankowsi & Møller 1995; Kodric-Brown *et al.* 2006). Interestingly, differences in the width of the baculum are found between dominant and subordinate males. Further experiments should test if the morphology of the baculum can explain the better performance of dominant males in post-copulatory competition (Klemme *et al.* 2006a).

Despite a high phenotypic variation, the length of the penile spines does not exhibit a positive allometry. Although the morphology or size of genitalia is generally unknown before the dissections of the individuals, which limits the possibilities in terms of experimental design, Dixson (1991) used a simple technique based on thioglycollate cream to remove spines from male marmosets (*Callithrix jacchus*) which appeared to be successful. The use of a similar technique to test the role of penile spines in bank voles could provide useful information. Finally, mammalian genitalia are studied with two-dimensional methods but some new progress in the understanding of genitalia function could come from the use of three-dimensional techniques (McPeek *et al.* 2009; Shen *et al.* 2009).

Chapter 4: Sperm allocation under assigned roles in the bank vole

4.1 Chapter overview

When females mate multiply in the same reproductive bout, sperm from different males are in competition to fertilize a set of ova. This sperm competition is an important evolutionary force that shaped different aspects of male phenotype such as sperm physiology and copulatory behaviour. Models of sperm competition generally assume that males have a finite quantity of resources to allocate to reproduction and that they have to share these resources between investment in gaining mating (i.e. pre-copulatory competition) and ejaculate expenditure (i.e. post-copulatory competition). It is generally predicted that males mating in a disfavoured role should invest more in sperm competition than males mating in a favoured role. I tested this hypothesis in the bank vole (*Myodes glareolus*). This species is particularly suited for this test since sperm competition is high due to female propensity to mate multiply and because males form social dominance relationships including dominant males (i.e. favoured males) and subordinate males (i.e. disfavoured males). For the first time in mammals, I investigated differences between dominant and subordinate males at three levels: relative sperm expenditure, ejaculate quality and investment in copulatory behaviour. Contrary to our predictions, I found that dominant males invested more in sperm competition through a higher number of sperm per ejaculate and a higher number of intromissions prior to ejaculation. However, sperm quality (assessed through sperm motility) and number of ejaculations was not different between dominant and subordinate males. These results differ from those reported previously for other taxa and emphasize the complexity of relationships between pre- and post-copulatory competition.

4.2 Introduction

(a) Sperm allocation

Sperm competition is a widespread phenomenon where sperm from two males or more are in competition to fertilize a set of ova (Parker 1970; Smith 1984a; Birkhead & Møller 1998; **Chapter 1**). This competition is traditionally compared to a raffle where the male probability to win the competition is proportional to the number of sperm invested by this male during the copulation (Parker 1998). Nevertheless, sperm production can be costly (e.g. Van Voohries 1992) and the number of sperm that males can invest in reproduction is limited (Dewsbury 1982). After a reproductive event, males are therefore likely to suffer from sperm depletion (Dewsbury 1982a; Nakatsuru & Kramer 1982) which can limit their fertilization success in subsequent matings (Preston *et al.* 2001). Hence, it is predicted that males who experience sperm competition should allocate their sperm strategically (review in Parker 1998) in response to the risk (Parker *et al.* 1997) or intensity (Parker *et al.* 1996) of sperm competition (see a description of the risk and intensity model of sperm competition in **Chapter 1**). Models of sperm competition 'risk' and 'intensity' have found support in a large range of taxa (review in Wedell *et al.* 2002). For example, studies in the meadow vole (*Microtus pennsylvanicus*), a promiscuous rodent, support predictions for both sperm competition risk and intensity models (delBarco-Trillo & Ferkin 2004, 2006). Males from this species increase their sperm investment when the risk of sperm competition perceived through olfactory cues is increased (delBarco-Trillo & Ferkin 2004) but decrease their investment when the intensity of sperm competition increases (delBarco-Trillo & Ferkin 2006), as predicted by the models (Parker *et al.* 1996, 1997). The same pattern of sperm investment in response to variation in the risk of sperm competition is found in Norway rats (*Rattus norvegicus*) (Pound & Gage 2004). However, in some species, the sperm allocation pattern can be different such as in house mice (*Mus musculus domesticus*) where males reduce their sperm allocation per ejaculate (Ramm & Stockley 2007) but increase the number of ejaculations (Preston & Stockley 2006) when the risk of sperm competition is enhanced by the presence of a rival male. Indeed, copulatory behaviour may also vary adaptively according to the level of sperm competition (Gomendio *et al.* 1998; Simmons 2001; Stockley & Preston 2004; **Chapter 1**). In rodents for example, the rate of intromissions is positively correlated with the level of sperm competition whereas the ejaculation latency is negatively correlated with it (Stockley & Preston 2004).

In addition to the quantity of sperm invested, several sperm characteristics play a role in male fertilization success (review in Snook 2005; Pizzari & Parker 2009). Traits such as sperm velocity (e.g. Gage *et al.* 2004; Malo *et al.* 2005a), length (e.g. Pitnick *et al.* 1995; Fitzpatrick *et al.* 2009) or viability (e.g. Hunter & Birkhead 2002; Fitzpatrick *et al.* 2009) can confer to males an advantage in sperm competition and these features constitute the sperm quality (Snook 2005). Nevertheless, compared to the well described effects of sperm number on male fitness (Wedell *et al.* 2002), it is much less clear how different sperm characteristics influence fertilization efficiency and differ between species (review in Snook 2005; **Chapter 1**). For example in cichlid fishes, sperm length is positively correlated with the level of sperm competition (Fitzpatrick *et al.* 2009; see also LaMunyon & Ward 1998) whereas a negative relationship between these two variables is found in passerine birds (Kleven *et al.* 2007 see also Garcia-Gonzalez & Simmons 2007). Therefore, to obtain a complete picture of male sperm allocation strategy, it is important to assess sperm expenditure in terms of both sperm number and quality. In a broader context, this will also help us to understand the evolution of male alternative post-copulatory strategies and their links with the energetic constraints that operate on male sperm production (Birkhead *et al.* 2009).

(b) Sperm allocation, copulatory behaviour and male social status

In species with dominance relationships, dominant males generally have a higher reproductive success than subordinates (Widdig *et al.* 2004). The better success generally found for these males can come from an advantage in pre-copulatory sexual selection (Cowlishaw & Dunbar 1991) and in post-copulatory sexual selection through sperm competition (Fitzpatrick *et al.* 2006) or cryptic female choice (Pizzari & Birkhead 2000).

In sperm competition games, the raffle can be unfair with some males mating in a favoured role and some males in a disfavoured role (Parker 1990a; Parker 1998). For example, in mammals, males who mate with a female close to the time of ovulation will be in a favoured role to fertilize her ova (Gomendio *et al.* 1998; **Chapter 1**). Since dominant males have an advantage in female monopolization (Cowlishaw & Dunbar 1991; van Noordwijk & van Schaik 2004), they are considered to be more often in a favoured role, whereas subordinates are more likely to be in a disfavoured role. These roles are generally assigned in a dominant – subordinate system (Dewsbury 1982b; Drews 1993; Parker 1990a). Theoretical predictions

related to the strategy that dominant and subordinate males should adopt to maximize their fitness have been proposed (Parker 1990a,b). Assuming that males trade-off between their investment in pre-copulatory sexual competition and post-copulatory sexual competition, it is predicted that subordinate males should expend more on sperm in a reproductive event than dominant males (Parker 1990a; Parker 1998). These predictions are supported in the Arctic charr (*Salvelinus alpinus*) (Rudolfsen *et al.* 2006). In this species, subordinate males that are in a disfavoured role (since they will release their sperm far from the females eggs compared to dominants) have a higher spermatocrit of ejaculates (Rudolfsen *et al.* 2006) and a higher initial sperm velocity in the fastest sperm cells (Haugland *et al.* 2008). Males are therefore able to adjust the quality of their ejaculate in relation to their social status (Rudolfsen *et al.* 2006) by producing for example faster sperm, which is a key factor in the fertilization success of externally fertilizing species (Gage *et al.* 2004) when they mate in a disfavoured role. However, a relationship between sperm quality and social status is not always evident (e.g. Montrose *et al.* 2008), and in some cases a positive relationship may be found, where high quality males have higher sperm expenditure than low quality males (e.g. Fox *et al.* 1997; Faulkes & Bennett 2001). In house mice, for example, sperm from dominant males are more motile than sperm from subordinate males, and this difference in sperm quality probably occurs after the establishment of social dominance (Koyama & Kamimura 1999; see also Koyama & Kamimura 2000).

Although there is some evidence that males adjust their ejaculate expenditure in relation to their social status (e.g. Rudolfsen *et al.* 2006), information concerning putative copulatory behaviour adjustment is generally lacking. However, previous studies reveal that in house mice, dominant males show more mounts and intromissions than subordinate males and the latency to the first mount, first intromission and first ejaculation is significantly shorter (deCatanzaro & Ngan 1983), which can give an advantage in sperm competition (Stockley & Preston 2004). Similarly, in montane voles (*Microtus montanus*), a species with a high level of sperm competition, dominant males perform significantly more thrusts than subordinate males (Shapiro & Dewsbury 1986). In this species, both dominant and subordinate males decrease their intromission and their thrust frequency after establishment of the social hierarchy (Shapiro & Dewsbury 1986). Unfortunately, these two studies do not provide information on the quantity of sperm transferred during the copulation.

(c) Aim of the study

To my knowledge, there is currently no comparison of alternative sperm allocation strategies between dominant and subordinate male mammals taking into account the number of sperm invested, the sperm quality and the pattern of the total sequence of copulatory behaviour. The bank vole (*Myodes glareolus*) is an ideal model for these investigations since in this species, females mate multiply but unequivocally prefer dominant males to subordinate males (Horne & Ylönen 1996; Kruczek 1997). Dominant males are therefore favoured in sperm competition and subordinate males disfavoured. Females have direct benefits from mating with dominant males since they are likely to sire more offspring (Klemme *et al.* 2006a; Kruczek & Zatorska 2008) and because male social status is heritable in this species (Horne & Ylönen 1998; Oksanen *et al.* 1999). However, it is still unclear if the better reproductive success of dominant males comes from an advantage in sperm competition and/or female cryptic choice (Klemme *et al.* 2006a).

In this study, I assessed differences in post-copulatory behaviour between dominant and subordinate males. As described above, several elements such as sperm quality and copulatory behaviour may explain differences in male reproductive success. To tease apart these potential confounding effects, two separate experiments were conducted. In the first one, I compared copulatory behaviour and reproductive physiology of males. In the second test, attention was focused on sperm allocation strategies and sperm quality of dominant and subordinate males. On the basis of models of sperm competition (Parker 1990a; Parker 1998), I predicted that subordinate males should allocate more sperm and/or better quality sperm to females to counteract their disadvantage in male pre-copulatory sexual competition through an advantage in sperm competition.

4.3 Methods

(a) Subjects

Bank voles used in these two experiments were the adult F1 and F2 offspring of 29 wild-caught individuals trapped in Cheshire (UK) between January and May 2007 (see also **Chapter 2**). After weaning, animals were housed with siblings of the same sex in MB1 cages (40 X 23.5 X 20 cm, North Kent Plastic Cages Ltd., UK) containing substrate (Corn cod Absorb 10/14 substrate) and paper-wool bedding material (hereafter called bedding). Food

and water were provided *ad libitum* (LabDiet 5002). Animals were maintained on a reversed photoperiod (light: 16 hrs, dark: 8 hrs, lights on at: 5:00 pm), and at a temperature of 21 ± 1°C. All experiments were conducted during the dark phase. For purposes of identification, male subjects were PIT tagged. All males were paired to establish social dominance relationships (see **Section 4.3.b**) and females were housed individually for the duration of the experiment to avoid mixing their odours with conspecific female odours. Males were split in two groups (hereafter called 'group 1' and 'group 2'), each constituted of both dominant and subordinate males. At the end I obtained a sample size of $n = 14$ dominant-subordinate pairs for 'group 1' and $n = 12$ dominant-subordinate pairs for 'group 2'. Unfortunately, several males did not copulate or died at different stages of the procedure such as before mating or between mating and dissection, which explains variation in sample sizes in the analyses.

(b) Social dominance establishment

I set up pairs of unrelated males in MB1 cages divided in two by a mesh barrier, with one male of each pair housed in each half of the cage (20 X 11.75 X 20 cm, North Kent Plastic Cages Ltd., UK). This separation allowed continuous olfactory, visual and auditory contact between males while they were housed together until the end of the experiment (**Figure 2.1**). To identify unambiguously dominant and subordinate individuals within each pair, I assessed male urinary marking behaviour, which is a technique commonly used to determine dominance relationships in bank voles (Rozenfeld *et al.* 1987; Horne and Ylönen 1996; Klemme *et al.* 2006a; see **Chapter 2**). To assess urine marking behaviour, both males from a pair were transferred into a clean benchkote-lined MB1 cage (again divided in two by a mesh barrier; **Figure 2.1**) and left for 30 min (in the dark phase period). Scent marks were scanned using a Multi-Image Scanner and analyzed using Scion image software (Biorad Fluor-S Multi-Image Scanner). The social status of the male was assessed based on the criteria identified by Rozenfeld and Rasmont (1991), with thin streaks of urine throughout the arena characteristic of dominant males, and large puddles of urine, especially in the corner of the arena, typical for subordinates (**Figure 2.2**). Before using males for the experiment, I waited to obtain this clear difference in scent marking within paired males on three consecutive occasions (with recovery of scent marks separated by at least 24 hours). These experimental males remained paired as described above over a period of 10 to 30 days after dominant-subordinate relationships were established.

(c) Experimental procedure

Males from 'group 1' were used for an experiment designed to compare differences in copulatory behaviour between dominant and subordinate males. Due to the difficulty of changing male roles after dominance assignment, I did not conduct a within-subject design as other studies sometimes have (e.g. Pound & Gage 2004; Rudolfsen *et al.* 2006). Males from this experiment had the opportunity to mate with a female to satiety (see **Section 4.3.i**). At the end of the copulation, females were housed back in their original cages. They were next checked for pregnancy and their litter size was noted to compare the reproductive success of dominant and subordinate males.

Males from 'group 2' were used to compare differences in sperm investment between dominant and subordinate males. F2 males from the original population were used to conduct this experiment. These males also had the opportunity to mate with females, but the copulation was stopped after the first ejaculation (see **Section 4.3.i**). Females were euthanized after the male's first ejaculation using halothane and by dislocating the neck.

After their mating trial, all males 'group 1' and 'group 2' were housed back in their original cages. They were euthanized using the same method as described above at least 1 week after they mated. This delay allows males to replenish their sperm reserves before the epididymal sperm count and sperm quality measures were performed (see **Section 4.3.f**; **Section 4.3.h**)

(d) Collection of preputial glands, seminal vesicles and testes

At the end of the two experiments performed in this study, males from both groups were sacrificed and several morphological measures were taken. All males were weighed (to the nearest 0.001 g) and measured using an electronic calliper (to the nearest 0.01 mm). Males were dissected and all paired preputial glands (hereafter called 'preputial gland'), seminal vesicles (hereafter called 'seminal vesicle'), testes (hereafter called 'testis') and epididymides (hereafter called 'epididymis') were weighed using an electronic balance (to the nearest 0.001 g).

Preputial gland weight is a useful way to confirm for dominant-subordinate relationships after the experiment, since it is a reliable indicator of male social status (Kruczek 1997; Łopuch & Radwan 2009).

(e) Collection of urine samples

Increased expression of urinary protein is generally an indicator of renal disfunctioning (Guder & Hofman 1993). However, some proteins such as major urinary proteins (MUP_S) (members of the licopalin superfamily) produced by the liver are small enough to be excreted in urine (Humphries *et al.* 1999). In the past two decades several studies have emphasized the complexity and the critical role of MUPs in sexual and social communication in house mice (*Mus musculus domesticus*) (e.g. Hurst *et al.* 2001; Novotny 2003; Cheetham *et al.* 2007). However, despite the fact that lipocalins are produced by bank voles (but not MUPs), little is known about the role of urinary proteins in this species (but see Turton 2007) and how the concentration in these proteins can vary among males of different social status. I collected urine from dominant and subordinate males to look for urinary protein concentrations. These measures were performed before and after males' social status was determined, therefore allowing urine concentration comparisons before and after the pairing. Bank voles were placed on a grating in a MB1 cage for one hour. Urine samples were collected and placed in a freezer for subsequent analysis. I followed the analysis protocol described by Cheetham *et al.* (2009) for mice. To correct for urine dilution, I measured the ratio of protein to creatinine (both expressed as mg/ml), because the quantity of creatinine (a by-product of muscle metabolism excreted in urine) is proportional to body mass. I measured the protein concentration of each sample using the 'Coomassie plus' protein assay reagent kit from Pierce, UK. I obtained the standard curve from a stock solution of bovine serum albumin (BSA) (1 mg/ml diluted to the range 0-50 μg/ml with ddH_2O). On a 96 well microtitre plate, I pipetted in duplicate 100 μl aliquots and added 250 μl of Coomassie. The absorbance of each sample was read at 595 nm in a Labsystems iEMS-MF plate reader and concentration was obtained from interpolation on the standard curve. Urine creatinine concentrations were obtained using the alkaline picrate assay from Sigma Chemicals, UK, and the standard curve was generated from a stock solution of creatinine (3 mg/dl diluted to the range 0-30 μg/m with ddH_2O). Each appropriately diluted sample was added in duplicate to a 96 well microtitre plate with 150 μl picrate reagent (5 ml picrate mixed with 1 ml sodium hydroxide).

The absorbance of each sample was read at 492 nm in a Labsystems iEMS-MF plate reader and concentrations were obtained from interpolation on the standard curve.

(f) Sperm collection from epididymis

I followed the same protocol that Ramm & Stockley (2007) used for mice to evaluate sperm reserves of males from 'group 1', which consists of dissection of the right epididymis of the euthanized males, followed by macerating the head and body of the epididymis with a scalpel blade for 1 min in 1 ml 1% citrate solution in a Petri dish. Sperm counts were performed on an Improved Neubaer haemocytometer using standard protocols (European Society of Human Reproduction and Embryology 2002).

(g) Sperm and copulatory plug collection from females

Before the start of the dissection, females that had just mated with males from 'group 2' were weighed and measured. To collect and count sperm from the female tract, I followed the same protocol as Ramm & Stockley (2007). After abdominal incision of the female, oviducts were clamped to prevent sperm migration. The tract was opened via a longitudinal incision down each uterine horn and placed in a Perspex Sterilin tube containing 1 ml 1 % citrate solution. I next agitated the tube for 5 minutes to let sperm disperse from the oviduct. Sperm counts were performed on an Improved Neubaer haemocytometer using standard protocols (European Society of Human Reproduction and Embryology 2002). Female copulatory tracts were then frozen in order to keep them intact. Several weeks later, these samples were defrosted and gently dissected to remove and weigh the copulatory plug using an electronic balance (to the nearest 0.001 g).

(h) Sperm quality

After taking the usual morphological measures of males from 'group 2' (see **Section 1.3.d**), I dissected their left epidymis and isolated their cauda. The cauda was next placed in a Petri dish containing 150 µl of B.W.W (Biggers Whitten Whittingham) medium solution. This solution, used to conserve sperm motility in mammals (e.g. Koyama & Kamimura 1999), contains 110 mM NaCl, 2 mM $CaCl_2$, 2.8 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 5 mM glucose, 1 mM sodium pyruvate, 20 mM sodium lactate, 4 mg/ml

bovine serum albumine (Sigma Co.) and 10 mM HEPES-NaOH (pH 7.4; 37 ± 1°C). Using a scalpel blade I performed 10 incisions in the cauda and let the sperm disperse for 1 minute. I then added another 250 µl of B.W.W. All 400 µl of B.W.W as next moved to an eppendorf tube in a water bath at 37°C until the dissection was finished (approximately 5 minutes). Ten µl of the solution contained in the eppendorf tube were put on a microscope slide and covered with a cover slip. The slide was next placed under the microscope (Leica DM1000 Microscope) on a microstat heated stage (Brunel Microscopes) set up at 37°C. Several videos of sperm motility were then recorded during 2 seconds (75 frames per second, 150 frames in total for each video) at magnification X10 and X20 using a Pointgrey camera (FL2-03S2M-C), no more than 20 minutes after the start of the dissection. Videos were analysed using Computer-Assisted Sperm Analysis (CASA) (Wilson-Leedy & Ingermann 2007), a Plugin implemented under ImageJ software (version 1.38x, http://rsbweb.nih.gov/ij/). Using this software, I first inverted the images, then adjusted the brightness and the contrast and finally adjusted and converted the threshold in order to select only the sperm heads for analysis. Several measurements were recorded: (1) curvilinear velocity (VCL, in µm/s) which estimates the velocity point to point along the trajectory (2) average path velocity (VAP, in µm/s) which estimates the point to point velocity over a constructed smooth path, and (3) straight line velocity (VSL, in µm/s) which estimates the velocity point to point along a straight line (**Figure 4.1**). The threshold values for excluding static sperm were set up as 25 µm/s for the VCL, 20 µm/s for the VAP and 3 µm/s for the VSL. The percentage of motile sperm (% motility) defined as the percent of sperm moving in a manner fitting motility determination parameters and the linearity (LIN = VSL/VAP) describing the path curvature were also calculated by the CASA Plugin (Wilson-Leedy & Ingermann 2007). For each individual, I recorded two videos. First I analysed each video twice to test for a repeatability of the measures taken by CASA Plugin on the same video. These measures were highly repeatable for each variable (e.g. for VAP: intra-class coefficient correlation: $r = 0.84$; $F_{35} = 11.61$; $P < 0.001$) and were therefore averaged. Next, I tested the repeatability of the measures between the two different videos recorded on the same individual using the average value of each video previously calculated. These measures were also highly repeatable (e.g. for VAP: intra-class coefficient correlation: $r = 0.82$; $F_{17} = 24.97$; $P < 0.001$) and were therefore averaged to obtain a mean value of each sperm quality traits investigated.

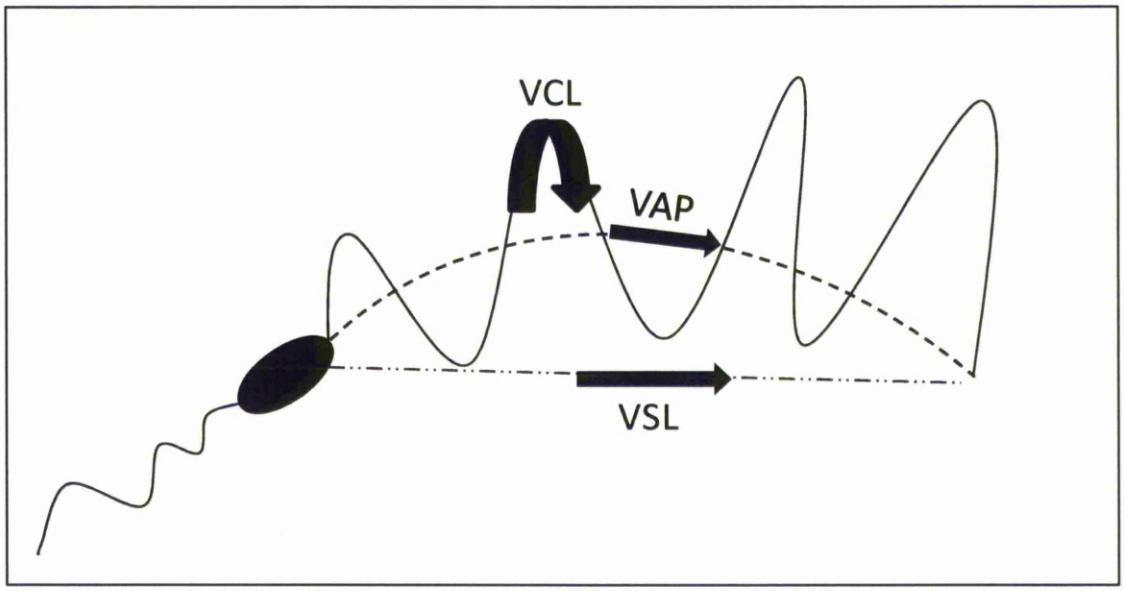

Figure 4.1 Measures recorded to analyse sperm motility (modified from Malo *et al.* 2005b). The curvilinear velocity (VCL) represents the total distance travelled by the sperm (point to point) per second. The velocity average path (VAP) represents the point to point distance travelled by a sperm on a path constructing a roaming average (calculated by CASA) per second and the velocity straight line (VSL) measured the distance travelled by the sperm between the first point and the furthest point from this origin during the measured time period following a straight line (Wilson-Leedy & Ingermann 2007).

(i) Mating trials

Each male from both groups was placed with an unrelated female in a neutral arena (70 x 60 x 50 cm). Males were placed first in the arena. I next added the female to the arena using a Perspex tube (diameter = 5 cm) closed at one end by a mesh barrier. I put the open end of the tube against one of the walls of the arena for 10 minutes in order to allow individuals to acclimate to each other through only visual and olfactory contact. The tube was then removed and females were in direct contact with males. If no mating behaviour occurred (mounts or intromissions) during the first 30 min of the experiment, or if persistent aggression occurred between the two individuals, the female was removed and replaced by a new unrelated female (see Klemme *et al.* 2006a). All copulations were recorded on a DVD using a CCTV video stream relayed to an adjoining room allowing rapid intervention in case of aggressive behaviour.

I separated pairs 30 min after the male's last ejaculation, since it is commonly found that a male who is sexually inactive for 30 minutes in a copulation series has mated to satiety (Dewsbury 1975; Stockley & Preston 2004). Since females were sexually naïve, male bedding was introduced daily into female cages for two days preceding their first mating opportunity to give them experience of a male odour. Dominant and subordinate males did not differ in the number of opportunities they had to mate with a female before they achieved a copulation for the first time (dominant: $\overline{X} = 6.43 \pm 1.45$; subordinate: $\overline{X} = 6.45 \pm 1.57$; $t_{23} = -0.01$; $P = 0.99$). In the second group of males, the pair was separated immediately after the first ejaculation. Females were then culled and dissected to count sperm no more than 15 minutes after ejaculation (see **Section 4.3.g**). As previously, dominant and subordinate males did not differ in the number of mating opportunities they had before achieving a copulation for the first time (dominant: $\overline{X} = 2.18 \pm 0.78$; subordinate: $\overline{X} = 4.25 \pm 2.59$; $t_{17} = -0.87$; $P = 0.40$). Three males did not transfer any sperm during the copulation and were allowed to mate a second time, at least one week after the first mating. Matings with no sperm transfer were excluded from the analysis.

(j) Analysis of male copulatory behaviour

At the end of all mating trials, male and female behaviour recorded on DVDs was analysed. As described in **Chapter 1**, bank vole copulatory behaviour is generally described as several ejaculation series, each one consisting of mounts (no vaginal penetration), intromissions (vaginal penetration without sperm transfer) and one ejaculation (sperm transfer). DVDs were watched in real time using the software Cyberlink Power DVD. The different parameters recorded are summarized in the **Table 4.1**.

Table 4.1 Variables used to measure male and female behaviour during copulation. Groups of males used to assess each of these variables are presented in the column 'group'.

Variables	Group	Complementary information
Number of intromissions in the first ejaculatory series	1 & 2	The first ejaculatory series is defined as the sequence between the first intromission and the first ejaculation
Number of mounts in the first ejaculatory series	1 & 2	
Number of intromissions in the total copulatory sequence	1	The total copulatory sequence is defined as the copulatory sequence between the first intromission and the last ejaculation
Number of mounts in the total copulatory sequence	1	
Intromission rate	1	Number of intromissions per minute during the first ejaculatory series.
Mount rate	1	Number of mounts per minute during the first ejaculatory series
Number of ejaculations in the total copulatory sequence	1	
Number of male approaches	1 & 2	Number of male copulatory attempts before the first mount or intromission (this is a good proxy of female resistance since a male fails to mount a female when she runs away from him)
Number of female approaches	1 & 2	Number of female approaches to the male between the start of the experiment and the first intromission (this variable measures the female interest to the male)
Intromission latency	1 & 2	Time between the start of the experiment and the first intromission
Ejaculation latency	1 & 2	Time between the first intromission and the first ejaculation (this corresponds to the duration of the first ejaculatory series)
Refractory period	1	Time between the end of an ejaculatory series and the start of the following series (i.e. next intromission)

(k) Statistical analysis

I assessed the normality of the data using Kolmogorov-Smirnov tests and when necessary I improved it by logarithmic transformations. If the normality of the data was not improved by log-transformation, I used non-parametric statistics instead. The homoscedasticity of the variance was assessed by Levene's test for equality of the variances. Comparisons between dominant and subordinate males were performed using independent *t*-test. However, when the homoscedasticy of the variances was not respected, I used the unequal variance *t*-test advocated by Ruxton (2006). All tests were two tailed and performed under SPSS 16.0. Data are presented as means ± standard error to the mean (s.e.m) and differences were regarded as statistically significant at $P < 0.05$.

4.4 Results

(a) Body mass and size

As previously reported in bank voles (Kruczek 1997; Klemme *et al.* 2006a), I found no difference in body mass or body length between dominant and subordinate males (body mass: $t_{44} = 1.13$; $P = 0.26$; body length: $t_{44} = 1.14$; $P = 0.26$; **Table 4.2**; **Figure 4.2**).

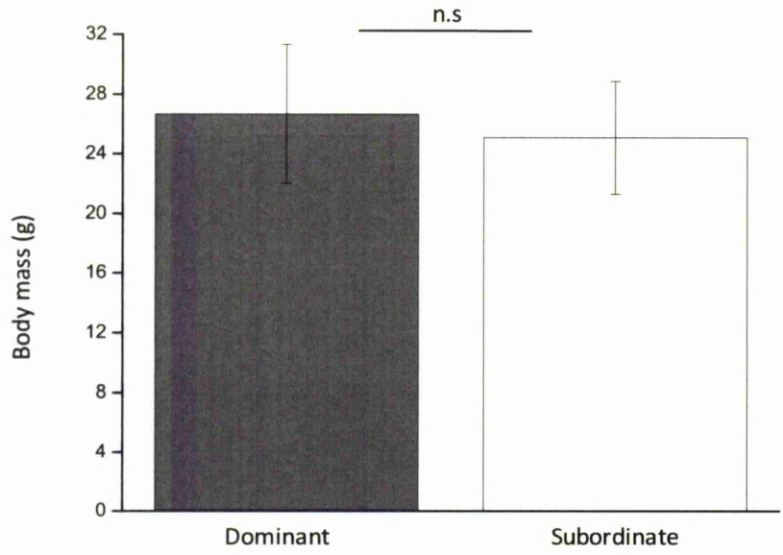

Figure 4.2 Mean body mass ± s.e.m (g) of dominant and subordinate male bank voles. No significant difference is found between the body mass of dominant and subordinate males ($t_{44} = 1.13$; $P = 0.26$).

(b) Preputial gland mass

Since preputial gland mass was correlated with body mass (n = 76; r = 0.43; $P < 0.01$), I compared the mass of the preputial gland between dominant and subordinate males using a GLM with preputial gland mass as the dependent variable, social status (dominant versus subordinate) as a fixed factor and body mass as a covariate. This confirmed that dominant males have significantly larger preputial glands that subordinate males ($F_{1,45}$ = 21.03; $P <$ 0.001) (**Table 4.2**; **Figure 4.3**). The dominant/subordinate classification based on scent mark behaviour in this study was therefore valid, since high social rank is associated with large preputial glands in rodents (Gustafsson *et al.* 1980; Kruczek 1997; Pohorecky *et al.* 2008).

(c) Seminal vesicle mass

Similar to the preputial gland, seminal vesicle mass was strongly correlated with body mass (n = 46; r = 0.54; $P < 0.001$), and so body mass was introduced as a covariate in a GLM analysis. This revealed that dominant males have significantly larger seminal vesicles for their body size compared to subordinates ($F_{1,45}$ = 6.77; P = 0.01) (**Table 4.2**; **Figure 4.3**).

(d) Testis and epididymis mass

Both testis and epididymis were strongly correlated with body mass (testis: n = 46; r = 0.68; P < 0.001; epididymis: n = 46; r = 0.52; $P < 0.001$; **Table 4.2**; **Figure 4.4**). After control for body mass, I found that dominant males have significantly larger testis and epididymis (testis: $F_{1,45}$ = 7.3; P = 0.01; epididymis: $F_{1,45}$ = 9.31; $P < 0.01$). Also, testis and epididymis mass were strongly correlated (n = 46; r = 0.72; $P < 0.001$).

Table 4.2 Summary of descriptive statistics for different biological traits of dominant and subordinate male bank voles. All these traits are compared with t-tests on log-transformed values (note that in the text differences in organ masses are tested using a GLM since the mass of each organ is correlated with body mass but results are qualitatively unchanged). For all paired organs, the reported values are the combined mass of the right and left part. * = $P < 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

		N	Minimum	Maximum	Mean	SEM	t-value
Body Mass (g)							
	Dominant	24	16.15	35.50	26.66	0.95	1.13
	Subordinate	22	18.54	30.85	25.09	0.81	
Body Length (mm)							
	Dominant	24	90.06	109.34	98.80	1.11	1.41
	Subordinate	22	84.46	105.50	96.99	1.09	
Preputial gland (mg)							
	Dominant	24	12.50	118.60	46.65	4.78	4.75***
	Subordinate	22	7.00	47.70	23.24	2.18	
Seminal Vesicle (mg)							
	Dominant	24	162.50	451,20	278.10	13.69	2.87**
	Subordinate	22	122.10	311.40	223.99	12.85	
Testis (mg)							
	Dominant	24	519.00	879,70	724.50	17.58	2.80**
	Subordinate	22	386.20	847.80	643.35	22.45	
Epididymis (mg)							
	Dominant	24	116.50	183.70	149.97	4.58	3.27**
	Subordinate	22	81.00	168.80	127.95	4.89	

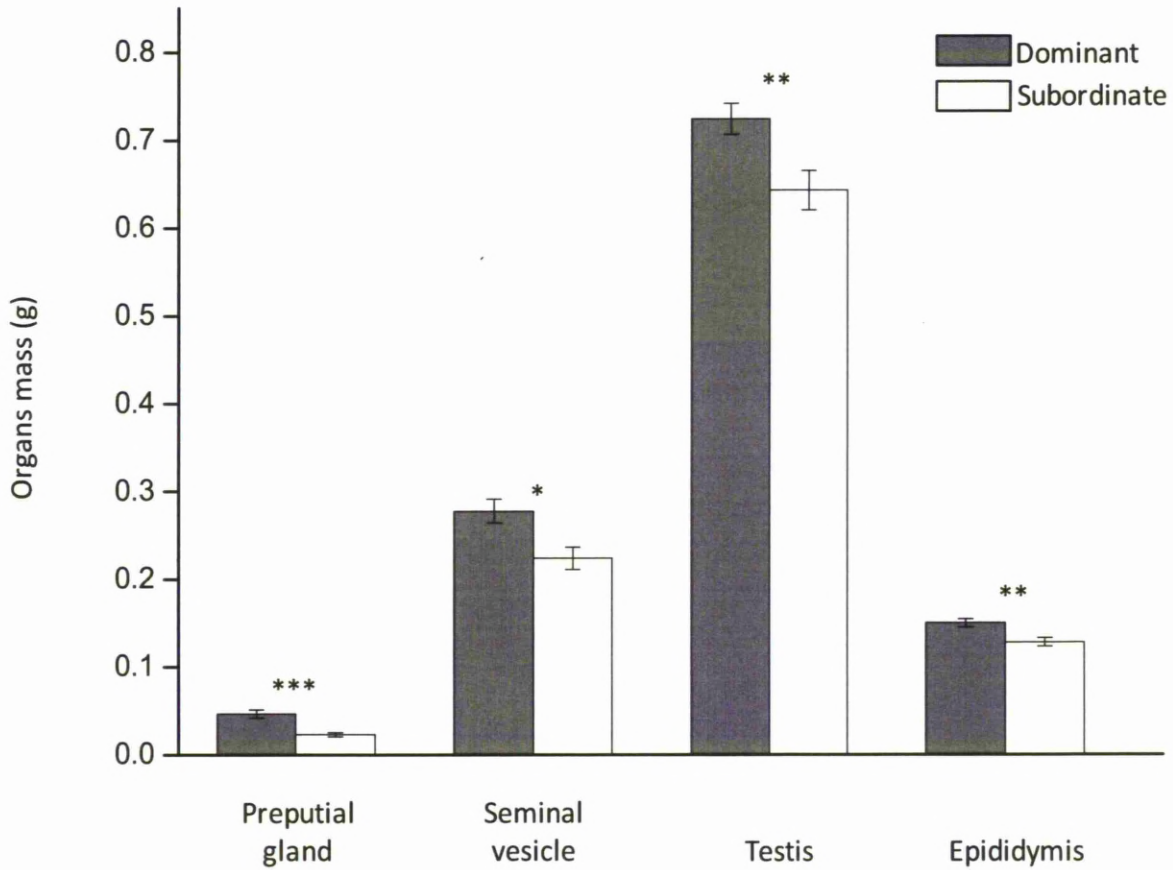

Figure 4.3 Differences in reproductive traits of dominant and subordinate male bank voles (mean ± s.e.m). Dominant males have significantly heavier preputial glands, seminal vesicles, testes and epididymides. * = $P < 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

(e) Urine concentration

Dominant males had a significantly higher protein concentration in their urine than subordinate males, both before and after the pairing (i.e. social dominance establishment) (before: $t_{13} = 3.70$, $P < 0.01$; after: $t_{16} = 2.23$, $P = 0.04$). However, when protein concentration were corrected for urine dilution (mg protein / mg creatinine) there were no difference in dominant and subordinate either before ($t_{13} = -0.52$, $P = 0.61$) or after ($t_{16} = -1.36$, $P = 0.2$) the pairing. Within the dominant males only, neither the concentration of protein, creatinine nor their ratio were significantly different before and after the pairing (protein: $t_5 = 1.39$, $P = 0.22$; creatinine: $t_5 = -0.58$, $P = 0.59$; ratio: $t_5 = 1.06$, $P = 0.34$). Similarly, none of these variables significantly differed before and after the pairing when subordinates were analyzed separately (protein: $t_7 = 1.6$, $P = 0.15$; creatinine: $t_7 = 0.75$, $P = 0.47$; ratio: $t_7 = 1.55$, $P = 0.16$). All concentrations are presented in **Table 4.3** and the range of protein concentration found is consistent with the results from Turton (2007).

Table 4.3 Concentration of protein and creatinine (mg/ml) in the urine of dominant and subordinate male bank voles. Dominant males have a significantly higher concentration of urinary protein before and after pairing (i.e. social dominance establishment) than subordinate males. * = $P < 0.05$; ** = $P \leq 0.01$

		Dominant	Subordinate	t-value
Before pairing				
	Protein mg/ml	1.79 ± 0.12 (N=7)	1.04 ± 0.16 (N=8)	3.70**
	Creatinine mg/ml	0.38 ± 0.04 (N=7)	0.20 ± 0.05 (N=8)	2.70*
	Ratio Protein/Creatinine	5.12 ± 0.80 (N=7)	5.72 ± 0.81 (N=8)	-0.52
After pairing				
	Protein mg/ml	1.44 ± 0.26 (N=6)	0.85 ± 0.13 (N=12)	2.23*
	Creatinine mg/ml	0.43 ± 0.09 (N=7)	0.18 ± 0.03 (N=12)	2.43*
	Ratio Protein/Creatinine	4.00 ± 0.97 (N=7)	5.70 ± 0.72 (N=12)	-1.36

(f) Litter size

There was no significant difference in the litter sizes produced following successful insemination by dominant or subordinate males (dominant: $\overline{X} = 3 \pm 0.57$; subordinate: $\overline{X} = 2.18 \pm 0.64$; $t_{23} = 0.95$, $P = 0.35$). Although 36 % of the matings (9 out of 25) did not lead to

pregnancy, there was no difference between dominant and subordinate males in their likelihood to initiate pregnancy (Fisher Exact Probability Test one-tailed: $P = 0.32$). The number of offspring produced was independent of male body mass ($n = 25$, $r = -0.01$, $P = 0.64$), testis mass ($n = 25$, $r = 0.30$, $P = 0.14$) and female body mass ($n = 25$, $r = 0.19$, $P = 0.37$).

(g) Epididymal sperm count

The number of sperm in the epididymis was strongly correlated with testis mass ($n = 25$, $r = 0.63$, $P < 0.001$) and there was no difference in the number of epididymal sperm between dominant and subordinate males using a GLM with 'testis mass' as a covariate ($F_{1,24} = 0.60$, $P = 0.44$). No difference was also found in the epididymal sperm count according to the male social status when 'testis mass' was removed from the model ($F_{1,24} = 0.008$, $P = 0.93$).

(h) Sperm allocation

Dominant males invested more sperm in their first ejaculation than subordinate males (dominant: $\overline{X} = 28.36 \pm 2.07 \times 10^6$; subordinate: $\overline{X} = 21 \pm 2 \times 10^6$; $t_{17} = 2.5$, $P = 0.02$) (**Figure 4.4**). Sperm investment was independent of female body mass ($n = 19$, $r = 0.16$, $P = 0.52$) and of male testis mass ($n = 16$, $r = 0.15$, $P = 0.58$) even though dominant males had heavier testis than subordinates ($t_{18} = 2.86$, $P = 0.01$).

(i) Sperm quality

All velocity measures (VCL, VAP and VSL) were strongly correlated ($n = 21$; coefficient of multiple correlation $r = 0.91$). There were no differences for any velocity variable investigated between dominant and subordinate males (**Table 4.4**). The percentage of motility and the linearity (LIN = VSL/VAP) which describes the path curvature were also not significantly different (**Table 4.4**). None of the velocity variables were correlated with testis mass (not shown).

Table 4.4 Summary of descriptive statistics for different sperm motility parameters and comparisons between dominant and subordinate male bank voles. No significant difference was found between dominant and subordinate males for each of these variables. The linearity (LIN) is the ratio VSL/VAP and describes the path curvature. The motility is the percent of sperm moving in a manner fitting motility determination parameters. (Wilson-Leedy & Ingermann 2007).

		mean	s.e.m	*t*	df	*P*
VCL (μm/s)						
	Dominant	185.91	16.64	-0.11	19	0.91
	Subordinate	189.67	16.29			
VAP (μm/s)						
	Dominant	101.05	12.48	-0.41	17.87	0.99
	Subordinate	108.51	16.11			
VSL (μm/s)						
	Dominant	81.07	9.76	0.01	17.16	0.99
	Subordinate	88.29	14.53			
LIN (%)						
	Dominant	80.22	1.76	0.35	19	0.73
	Subordinate	78.78	3.58			
Motility (%)						
	Dominant	50.62	7.20	0.25	19	0.80
	Subordinate	48.12	6.92			

(j) Copulatory plug allocation

Dominant and subordinate males did not appear to invest differently in the mass of the copulatory plug (dominant: $\overline{X} = 27.4 \pm 9.1$ mg; subordinate: $\overline{X} = 32.0 \pm 7.7$ mg; $t_{16} = -1.32$, $P = 0.21$) (**Figure 4.4**) although the seminal vesicles, which are involved in the production of the copulatory plug, were significantly heavier for dominant males (**Figure 4.3**). The mass of the copulatory plug was not correlated with the mass of the seminal vesicles ($n = 15$, $r = 0.001$, $P = 0.99$), with the number of sperm invested ($n = 18$, $r = -0.02$, $P = 0.94$) or with the female body mass ($n = 18$, $r = 0.02$, $P = 0.93$).

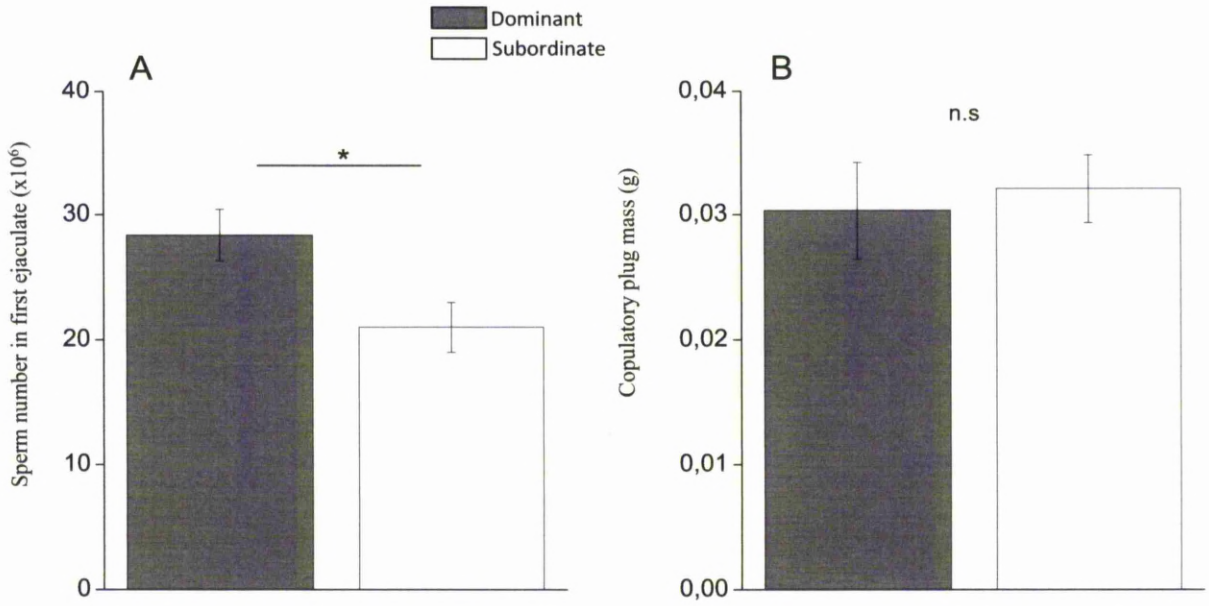

Figure 4.4 Differences in sperm number per ejaculate (A) and copulatory plug mass (B) between dominant and subordinate male bank voles (mean ± s.e.m). Dominant males invest significantly more sperm than subordinates ($t_{17} = 2.5$, $P = 0.02$) whereas no significant difference is found in the mass of the copulatory plugs produced ($t_{16} = -1.32$, $P = 0.21$).

(k) Copulatory behaviour

During the first ejaculatory series, dominant males perform significantly more intromissions than subordinate males (dominant $\overline{X} = 21.1 \pm 1.8$; subordinate $\overline{X} = 16.4 \pm 1.9$; $t_{29.2} = 2.12$, $P = 0.04$) **(Figure 4.5)** but there were no significant differences in the number of mounts achieved according to male social status (dominant: $\overline{X} = 10 \pm 1.9$; subordinate: $\overline{X} = 16.6 \pm 5.0$; $t_{42} = -0.40$, $P = 0.69$) **(Figure 4.5)**. Dominants and subordinates did not differ either in the time between the first intromission and the first ejaculation (dominant: $\overline{X} = 19.3 \pm 1.7$ min; subordinate: $\overline{X} = 16.8 \pm 2.8$ min; $t_{26.99} = 1.51$, $P = 0.14$) or the intromission rate (dominant: $\overline{X} = 1.25 \pm 0.11$ intromissions/min; subordinate: $\overline{X} = 1.45 \pm 0.20$ intromissions/min; $t_{42} = 0.04$, $P = 0.97$), but a marginally significant difference was found for the mount rate, higher for subordinates (dominant: $\overline{X} = 0.52 \pm 0.07$ mounts/min; subordinate: $\overline{X} = 1.12 \pm 0.26$ mounts/min; $t_{21.9} = -2.10$, $P = 0.05$).

The time between the start of the experiment and the first intromission (i.e. intromission latency) was not significantly different between dominant and subordinate males (dominant:

$\overline{X}$ = 6.7 ± 1.5 minutes; subordinate: $\overline{X}$ = 7.4 ± 1.4 minutes; t_{42} = -1.19, P = 0.24) **(Figure 4.5)**. During this period, males approached females regardless of dominance status (number of approaches by dominant: $\overline{X}$ = 9.1 ± 1.6; by subordinate: $\overline{X}$ = 9.4 ± 2.9; t_{42} = 0.80, P = 0.43) and females made no more approaches to dominant males than to subordinate males (number of female approaches: dominant $\overline{X}$ = 1.0 ± 0.4; subordinate $\overline{X}$ = 1.4 ± 0.5; t_{42} = -1.19, P = 0.24).

Analyses on males from 'group 1' allowed comparisons of copulatory behaviour between dominant and subordinate males when they were allowed to mate to satiety. If we consider the total copulatory sequence, dominant and subordinate males did not differ significantly in their number of mounts (dominant: $\overline{X}$ = 25.4 ± 5.4; subordinate: $\overline{X}$ = 45.1 ± 15.8; t_{23} = -0.52,; P = 0.61), intromissions (dominant: $\overline{X}$ = 48.4 ± 4.3; subordinate: $\overline{X}$ = 51.3 ± 3.5; t_{23} = -0.75, P = 0.46) or ejaculations (dominant: $\overline{X}$ = 3.4 ± 0.3; subordinate: $\overline{X}$ = 3.1 ± 0.3; t_{23} = 0.68, P = 0.50; **Figure 4.5**). As not many males performed more than 4 ejaculations, I compared the refractory period of dominant and subordinate males only until the third sequence. There was no significant difference between dominant and subordinate males in the time spent between the end of the first sequence and the start of the second (dominant: $\overline{X}$ = 722.7 ± 39.52 s; subordinate: $\overline{X}$ = 760.4 ± 83.3 s; t_{23} = 0.33, P = 0.75), or between the end of the second sequence and the start of the third sequence (dominant: $\overline{X}$ = 924.5 ± 78.5 s; subordinate: $\overline{X}$ = 802.6 ± 164.5 s; t_{16} = 0.47, P = 0.65). When dominant and subordinate bank voles were pooled together, the mean number of ejaculations was 3.24 ± 0.22 during a copulatory period of 4569.36 ± 313.45 s (76.16 min ± 5.22 min). This total duration of copulation was not correlated with male body mass (n = 25, r = -0.13, P = 0.52).

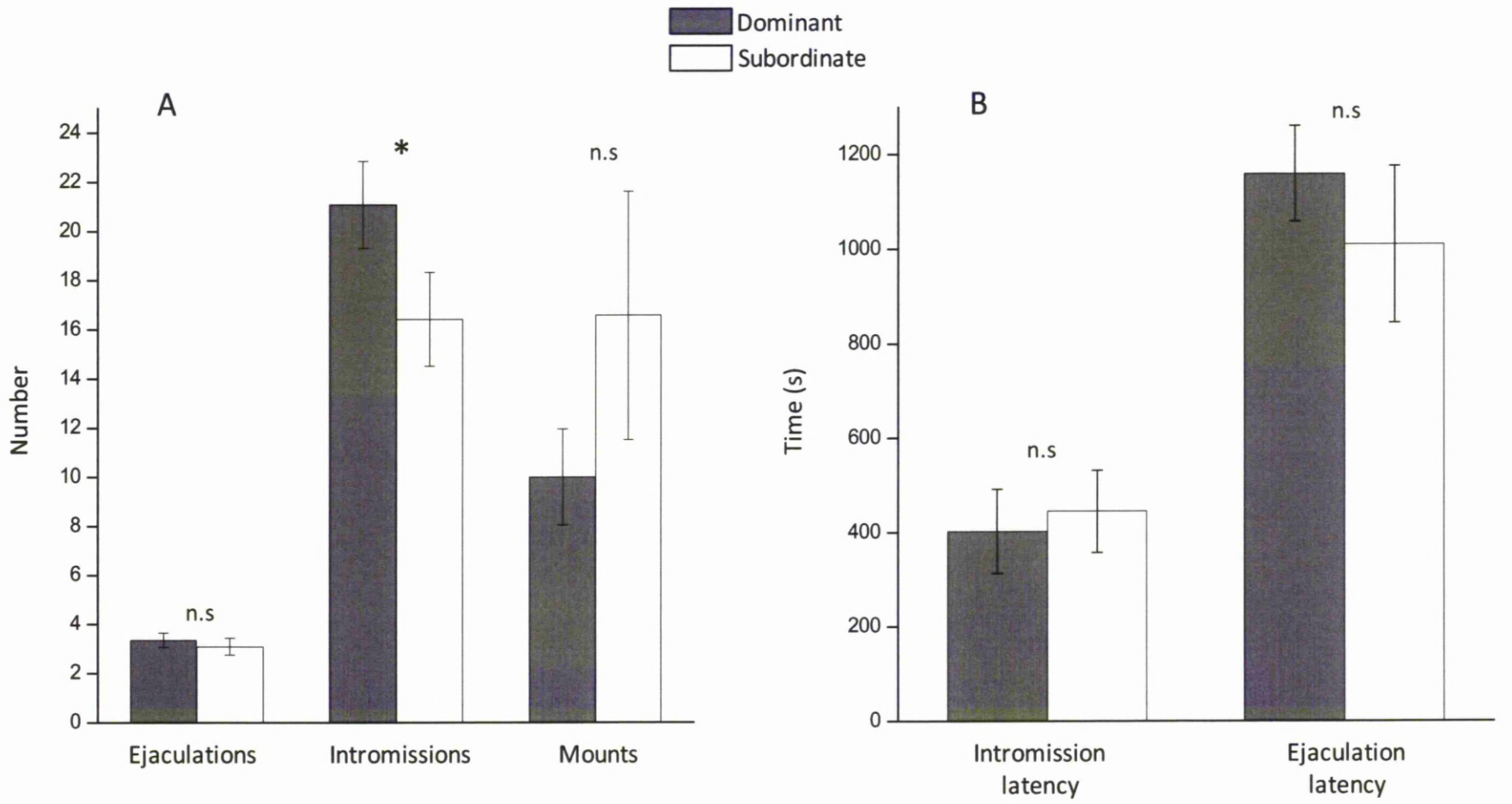

Figure 4.5 Comparison of copulatory behaviour for dominant and subordinate male bank voles (mean ± s.e.m). During the first copulatory sequence, dominant males perform significantly more intromissions than subordinate males ($t_{29.2} = 2.12$, $P = 0.04$) but the number of mounts during the first copulatory sequence and the number of ejaculations did not significantly differ according to male social status (see results section). Similarly, intromission latency (time between the start of the experiment and first intromission) and ejaculation latency (time between the first intromission and the first ejaculation) did not differ significantly between dominant and subordinate males.

4.5 Discussion

The aim of this study was to test if subordinate males invest more in post-copulatory competition than dominant males (Parker 1990a). Contrary to theoretical predictions, the results show that it is dominant male voles that invest more sperm per ejaculate. This contrasts with empirical evidence found in other taxa (e.g. Neff *et al.* 2003; Rudolfsen *et al.* 2006). Also, despite no difference found in the epididymal sperm count in relation to social status, dominant male voles have larger testes mass, consistent with the larger ejaculate found for these males (Møller 1988a; Møller 1989).

There was no difference in the number of ejaculations per female according to male social status. However, it seems that the first ejaculation plays a crucial role in male fertilization success since Klemme *et al.* (2006a) found higher reproductive success for dominant males when they are allowed to ejaculate once. Moreover, under natural conditions, males and females mate multiply over short periods (Ratkiewicz & Borkowska 2000) and are likely to be disturbed during the copulation. Investing the largest quantity of sperm in the first ejaculation might decrease the cost of failing to complete further ejaculations due to the presence of an intruder such as a competitive male or a predator. The absence of a relationship between the number of sperm invested and the number of ejaculations has been reported in meadow voles (delBarco-Trillo & Ferkin 2004) and may come from variation in vas deferens and/or cauda epididymis contractions (Pound 1999; Anderson *et al.* 2004; **Chapter 7**), which increases the number of sperm in the epididymis prior to the first ejaculation when a risk of sperm competition is perceived (delBarco-Trillo & Ferkin 2007a; but see Ramm & Stockley 2007). Moreover, although it is not the case in meadow voles (delBarco-Trillo & Ferkin 2007a), the presence of a female (or a female odour) might trigger an increase of the number of sperm in males' epididymis. In our experiment, males were killed one week after their mated and the fact that they had no contact with females during that week could explain why I did not find any differences in the epididymal sperm count of dominant and subordinate males.

As expected, preputial glands were heavier in dominant males. These glands produce compounds such as hexadecylacetate (Brinck & Hoffmeyer 1984) which are released in urine and involved in olfactory signalling (Viitala & Hoffmeyer 1985; Kruczek 1994). This result confirms that social rank determination based on scent marking behaviour was reliable.

Indeed, it is well established that these glands are costly to produce (Radwan *et al.* 2006), condition-dependent (Łopuch & Radwan 2009), and bigger in dominant compared to subordinate males (Gustafsson *et al.* 1980; Kruczek 1997; Koyama & Kamimura 2000; Pohorecky *et al.* 2008).

Before the first ejaculation, dominant males performed more intromissions than subordinate males, as previously reported by Horne and Ylönen (1996) in bank voles and deCatanzaro and Ngan (1983) in mice. Several hypotheses have been suggested to understand the benefits of performing a higher number of intromissions than required to initiate pregnancy (see Stockley & Preston 2004), such as an improvement of the ejaculatory performance (Toner & Adler 1986 but see Stockley & Preston 2004) or a reduction in the female propensity to remate (Huck & Lisk 1986). This second hypothesis seems seductive especially in bank voles since dominant males are particularly successful when they mate last (Klemme *et al.* 2006a; see also Dewsbury 1982b). Also, if males benefit from an increased number of intromissions, dominant males might spend more time stimulating females because they have less chance of being interrupted than subordinates. However, this hypothesis seems unlikely since I did not find differences in ejaculation latency in relation to male social status. Finally, it is important to acknowledge that the higher number of intromissions performed by dominant males might come from a female preference for mating with dominants. Indeed, if females are more resistant to the intromissions attempts of subordinate males it would explain why I found a higher number of mounts in subordinates (although not significant). It thus seems unlikely that an increased number of mounts gives an advantage in sperm competition as is the case in fowl (Løvlie *et al.* 2005) and more probably, subordinate male bank voles might have more difficulties controlling females and might fail in their intromission attempts more often, which leads to a higher mount rate. Nonetheless, before the start of the mating sequence, females did not show differential interest between dominant and subordinate males, which concurs with evidence that receptive females generally accept to mate with any male, regardless of his social status (Klemme *et al.* 2007a).

In our study, sperm motility did not differ significantly according to male social status (but see Kruczek & Styrna 2009 in this species). Differences in sperm allocation without difference in sperm quality are not uncommon. For instance, in the bluegill sunfish (*Lepomis macrochirus*), males with alternative mating strategies differ in their sperm allocation but not in their sperm motility (Stoltz & Neff 2006; see also Pattarini *et al.* 2006). However,

investigations of the relationship between sperm motility and dominance relationship generally report mixed results. For example, in fishes (Rudolfsen *et al.* 2006) and fowl (Pizzari *et al.* 2007), subordinate males produce more motile sperm whereas in mice, more motile sperm are found in dominant than in subordinate males (Koyama & Kamimura 1999) perhaps due to a suppression of subordinate males' sperm activity through hormonal processes (Koyama & Kamimura 1999). Therefore, a larger investment in sperm per ejaculate does not necessarily trade-off with a decline in sperm motility.

Similarly to Kruczek (1997), I found heavier seminal vesicle glands in dominant male bank voles. These glands are involved in the production of the copulatory plug (Ramm *et al.* 2005) but the investment in this plug did not differ significantly with social status in our study. However, seminal fluid proteins produced by seminal vesicle glands might also play a role in sperm competition through for example an allospermicide function (review in Poiani 2006). In bank voles, females show a preference toward dominant males (Horne & Ylönen 1996; Kruczek 1997) and dominant males have an advantage in intra-sexual contests (Kruczek 1997). This would allow dominant males to have a higher mating rate than subordinate males, which would in turn explain the larger testes found in dominant males (Parker & Ball 2005) but also the larger seminal vesicles since these structures coevolved in rodents (Ramm *et al.* 2005).

4.6 Conclusion

Contrary to theoretical predictions (Parker 1990a; see also Tazzyman *et al.* 2009), the results presented here show that subordinate male bank voles do not counteract their disfavoured role by investing more in ejaculate expenditure than dominant males. On the contrary, dominant males invested more in post-copulatory competition through a higher number of sperm invested per ejaculate and a higher number of intromissions. However, sperm quality did not differ according to the male social status in this study. Contrary to some other taxa, dominant males in bank voles appear to be therefore better competitors in both pre-copulatory sexual competition (Horne & Ylönen 1996; Kruczek 1997) and in post-copulatory sexual competition. This higher sperm allocation by dominant males might explain their higher reproductive success in competitive situation (Klemme *et al.* 2006a). In this study, contrary to Kruczek & Zatorska (2008) who used a larger sample size, I did not find differences in litter size according to male social status. However, under natural conditions females mate multiply

(Ratkiewicz & Borkowska 2000) and therefore potential differences in reproductive success between dominant and subordinate males have to be investigated in a competitive context (see Klemme *et al.* 2006a).

More generally, further studies should investigate more closely the relationship between pre- and post-copulatory sexual selection, especially how the investment in gaining mating (e.g. olfactory signalling in rodents) constrains male reproductive expenditures (see also Simmons & Emlen 2006). Also the paradox of female bank voles to promote copulation with subordinate males after mating with a dominant male (Klemme *et al.* 2006a) now deserve more attention since the investment in sperm per ejaculate by dominants is sufficient to ensure the fertilization of female eggs. These two points will be discussed more thoroughly in **Chapter 7**.

Chapter 5: Ejaculate production according to social experience in the bank vole

5.1 Chapter overview

Sperm competition theory predicts that males should strategically allocate larger ejaculate when the level of sperm competition is elevated. However, an increase in ejaculate expenditure generally requires adaptation in the structures involved in sperm and seminal fluid production (i.e. testes, seminal vesicles). Since ejaculate production is costly, it is predicted that males should adapt their investment to the population average level of sperm competition. Moreover, models of sperm competition generally assume that males have a finite quantity of energy devoted to reproduction and predict that they should trade-off between an investment in pre-copulatory competition and an investment in post-copulatory competition. In the present study, I investigated plasticity in ejaculate production and quality (i.e. sperm motility) in response to the population average level of sperm competition in the bank vole (*Myodes glareolus*). The level of sperm competition was manipulated by controlled exposure to the odour of rival males during sexual development. Male bank voles did not adjust their investment in sperm production and quality according to the average level of sperm competition. However, males that experienced a 'high' level of competition had larger seminal vesicles than males that experienced a 'low' level of competition. Male bank voles might benefit from enlarged seminal vesicles when the average level of sperm competition in the population is high, since these glands might permit a higher mating rate and/or promote male success in sperm competition. No support for a trade-off between an investment in scent marking and sperm production / quality was found in this study. More generally, these results contrast with recent findings for house mice and emphasize the importance of studying species with different social and mating systems to understand the costs and limits of phenotypic plasticity in reproductive traits.

5.2 Introduction

After an ejaculation, males generally require time to replenish their sperm reserves. This 'sperm depleted' state can temporarily limit male mating rate (Dewsbury 1982a; Preston *et al.* 2001; Lewis & Wedell 2007; **Chapter 1**). Therefore, males are predicted to allocate their sperm strategically in relation to the 'risk' or 'intensity' of sperm competition (Parker *et al.* 1996; Parker *et al.* 1997; Parker 1998; Parker & Ball 2005; **Chapter 1**). Recent compilations of evidence in a wide range of taxa supports this prediction (review in Wedell *et al.* 2002; see also Pound & Gage 2004; delBarco-Trillo & Ferkin 2006; Simmons *et al.* 2007). All else being equal, greater sperm expenditure per ejaculate implies that individuals are able to produce more sperm, often via an increase in relative testis size (Amann 1970; Møller 1988a; Møller 1988b; Møller 1989). It is now well documented through comparative studies across species that high levels of sperm competition are associated with large testes (relative to body size) (e.g. Hosken 1997; Byrne *et al.* 2003; Pitcher *et al.* 2005; Ramm *et al.* 2005) and more precisely, at least in New World Blackbirds, with a high proportion of seminiferous (sperm-producing) tissue within the testis (Lüpold *et al.* 2009). Also, the relative size of mammalian testes appears to be correlated negatively with the length of the cycle of the seminiferous epithelium (Ramm & Stockley 2010) which emphasizes the sperm competition advantages conferred by a higher rate of sperm production (Lüpold *et al.* 2009; Ramm & Stockley 2010).

Until now, intraspecific variation in sperm production in relation to average (or population) levels of sperm competition has received relatively little attention, and the majority of evidence comes from invertebrates (e.g. Gage 1995; Oppliger *et al.* 1998; Stockley & Seal 2001; Schärer & Ladurner 2003; Schärer & Vizoso 2007; Brauer *et al.* 2007 but see Ramm & Stockley 2009a). In the flatworm (*Macrostomum* sp.), a simultaneous hermaphrodite, individuals raised in larger groups have a higher sperm production rate, which is associated with bigger testes compared to individuals raised in smaller groups (Schärer & Ladurner 2003; Schärer & Vizoso 2007). Similarly, in the fruit fly *Drosophila bifurca*, males raised in large groups produce sperm at a faster rate compared to solitary males (Bjork *et al.* 2007) even if in this study other factors such as differences in number of mating opportunities and sex-ratio cannot be excluded in the interpretation of the results (Bjork *et al.* 2007). These examples suggest that phenotypic plasticity in sperm production according to the level of sperm competition might be common, at least among invertebrate taxa.

Other ejaculate components, such as seminal fluid proteins, can influence the outcome of post-copulatory sexual competition (Chapman *et al.* 2000; Fricke *et al.* 2009; see also Cameron *et al.* 2007). For example, in *Drosophila melanogaster*, males with larger accessory glands have a higher reproductive success in competitive situations since they can produce and transfer more sex-peptide (a protein contained in the seminal fluid that increases female egg production and decreases female receptivity) during copulation (Wigby *et al.* 2009). Moreover, male's investment in accessory glands varies with the level of sperm competition experienced (Crudgington *et al.* 2009). Indeed, in *Drosophila pseudoobscura*, males from highly promiscuous lines develop larger accessory glands (but not larger testes) compared to males from monogamous lines (Crudgington *et al.* 2009). Evidence for adaptive plasticity in accessory gland investment remains limited but its study is of interest to understand how males respond to variation in the level of sperm competition (Cameron *et al.* 2007).

To date, evidence for plasticity of ejaculate production in mammals has only been tested experimentally in house mice (*Mus musculus domesticus*). It has been shown that male mice in regular contact with three competitors had higher daily sperm production rates and higher numbers of sperm in the caput epididymis than males who regularly experienced the presence of one competitor only (Ramm & Stockley 2009a). However, this adaptive response to the average risk of sperm competition was independent of testis and seminal vesicle size (Ramm & Stockley 2009a; but see Long & Montgomerie 2006). The bank vole (*Myodes glareolus*) differs from house mice in social organization and mating system. Male bank voles occupy home ranges overlapping different female home ranges (Bujalska 1970; Bujalska & Saitoh 2000; **Chapter 1**) and females are likely to promote polyandry (Klemme *et al.* 2006a; Klemme *et al.* 2007a). Consequently, female multiple mating is common in natural populations (Ratkiewicz & Borkowska 2000) whereas in the house mouse the risk of competition varies according to population density but is lower overall (Dean *et al.* 2006).

In the present study, I investigated if male bank voles adjust their ejaculate production according to their social experience of rival males. I also aimed to test if investment in ejaculate production is limited by investment in pre-copulatory competition. Indeed, models of sperm competition (e.g. Parker 1990a; Parker 1990b) generally assume that males have a

finite quantity of resources devoted to reproduction and that they have to trade-off between their investment in gaining copulations (i.e. pre-copulatory competition) and their investment in ejaculate production (i.e. post-copulatory competition) (Parker 1998; see also Simmons & Emlen 2006). I quantified male investment in pre-copulatory competition through scent marking behaviour, since scent marking is costly (Roberts 2007) and since there is widespread evidence that females prefer males who invest extensively in scent marking (Horne & Ylönen 1996; Kruczek 1997). For the first time in mammals, I also investigated potential plasticity in ejaculate quality by measuring different parameters of sperm motility (see Methods) since there is now compelling evidence that sperm motility is an important aspect of male reproductive success (Gage *et al.* 2004; Malo *et al* 2005a; review in Snook 2005; review in Pizzari & Parker 2009).

5.3 Methods

(a) Subjects

Male subjects (n = 28) used in this experiment were the F3 offspring of 29 wild-caught individuals trapped in Cheshire (UK) between January and May 2007. They were kept with their parents in MB1 cages (40 X 23.5 X 20 cm, North Kent Plastic Cages Ltd., UK) until weaning at approximately 22 days and then were randomly assigned to two different experimental groups (see below). For the duration of the experiment, these males were housed individually in M3 cages (48 X 11.5 X 12 cm, North Kent Plastic Cages Ltd., UK) containing (Corn cod Absorb 10/14 substrate) and paper-wool bedding material (hereafter called bedding). Food and water were provided *ad libitum* (LabDiet 5002). In order to control male social status, pairs of cages were placed within high-sided enclosures (1.2 m X 1.2 m) (see below; **Figure 5.1**). Male subjects were PIT tagged for individual identification. An additional 8 sexually mature males, housed individually in M3 cages were used to manipulate the level of social experience of the male subjects. Similarly, 21 sexually mature females, housed as 7 trios of unrelated females in MB1 cages were used to provide female odours (see **Section 1.3.b**). The latter additional males and females were the adult F2 individuals from the same population and were housed in a separate room to the male subjects. In both rooms, animals were maintained on a reversed photoperiod (light: 16hrs, dark: 8hrs, lights on at: 5:00 pm), and at a temperature of 21 ± 1°C.

(b) Manipulation of social experience

I tested for an influence of perceived average (or population) level of sperm competition on sperm production by manipulating the social experience of recently weaned males over a period of 10 weeks. This period exceeds the duration of bank vole spermatogenesis of 31 ± 0.7 days (Grocock & Clarke 1976). After weaning, males were assigned to one of two experimental groups: a 'high' competition group (n = 14) or a 'low' competition group (n = 14). To test for a relationship between investment in sperm production and investment in scent marking, males with different reproductive effort in gaining matings were needed. Therefore, to increase the chance of observing opposite strategies in scent marking investment between males, I assigned two males to each enclosure (Horne & Ylönen 1996; Klemme *et al.* 2006a). These males were unrelated, housed in two different M3 cages (distance between cages: 30 cm) but in regular contact (see below). To summarize, 28 males were housed individually within 14 enclosures: 7 pairs assigned to the 'low' competition group and 7 pairs assigned to the 'high' competition group (**Figure 5.1**).

At the start of the experiment, individuals from the two treatment groups did not differ in their body mass ('low': $\overline{X}$ = 13.67 ± 0.51 g, 'high': $\overline{X}$ = 14.03 ± 0.42 g; t_{26} = 0.60, P = 0.55) or age ('low': $\overline{X}$ = 25.00 ± 0.59 days, 'high': $\overline{X}$ = 24.92 ± 0.57 days; t_{26} = -0.09, P = 0.93). To stimulate the development of normal sexual physiology and behaviour (Vandenbergh 1971), all males received regular exposure to female odours during the experiment. Each male received once a week (every Monday; **Table 5.1**; **Table 5.2**) 12.5 g of female bedding freshly removed from one of the eight female cages described above. Bedding from each female cage was sampled to be used in four different male cages, balanced between the two treatment groups (**Figure 5.1**). All males were unrelated to the females they received odour from.

In the 'high' competition group, males received contact with social odours of four different males every week: one male from the same enclosure and three 'external' sexually mature males. The three external males used to provide odours were kept in a separate room (see below; **Table 5.1**). In the 'low' competition group males were in contact only with the odour of one other male (i.e. the male present in the same enclosure). To provide contact with social

odours, subject males received in their enclosure, once a week, a small amount of bedding (approximately 12.5 g) collected in the cage of the other male. On three other days of the week (**Table 5.2**), males from the 'low' competition group received a small quantity of clean bedding (approximately 12.5 g) and males from the 'high' competition group received an odour from one of three external males (approximately 12.5 g; **Table 5.1**). In the 'high' competition group, males from the same enclosure received bedding from the same external males, on a weekly basis (**Table 5.1**). Because eight external males were used to provide odours to individuals of the 'high' competition group, these external males were used for several enclosures (maximum 3 enclosures) but the same combination of odours from three of these males was never used twice between each enclosure (**Figure 5.1**). Odour is an appropriate stimulus to simulate male competition since it conveys signals used for individual recognition in rodents (delBarco-Trillo & Ferkin 2004; Hurst & Beynon 2004; Hurst 2009; Ramm & Stockley 2009a). Since antagonistic contacts promote differential scent marking investment between two individuals encountering each other, it increased our chances to observe opposite patterns of urine deposition in each enclosure by releasing males simultaneously for 30 min once a week between week 2 and week 4, and sequentially once a week for 1 hour between week 5 and week 10 (**Table 5.1**; **Table 5.2**).

Unfortunately one of the males died on the first day of the third week of the experiment. This male was replaced by another male born the same week and housed alone in a M3 cage from weaning. This new male was unrelated to the other male in the enclosure.

(c) Scent marking investment

To assess scent marking behaviour, both males from the same enclosure were transferred during the dark phase to clean benchkote-lined MB1 cages divided in two by a mesh barrier allowing olfactory and visual contact (see also **Chapter 2**). Males were left for 30 min. Scent marks were collected during the last week of the experiment over 3 consecutive days and the number of scent marks was highly repeatable across these 3 days (repeated-measures ANOVA; $F_{1,27} = 0.03$; $P = 0.87$). In order to familiarise subjects with the apparatus, 2 scent marking trials (achieved with the same design as described above) were performed during week 6 of the experiment. After scent marks were scanned using Bio-rad Fluor-S^{TM} MultiImager (QuantityOne software: 12sec exposure, 530DF60 Filter, UV light source Epi illumination, high resolution mode) (see also **Chapter 2**), the number of scent marks was

quantified by analysing images with the 'Analyze Particles' tool in ImageJ (version 1.38x, http://rsbweb.nih.gov/ij/). Using this software, colours were inverted, and brightness, contrast and threshold were adjusted in order to avoid scent marks miscounting.

(d) Reproductive morphology: preputial glands, seminal vesicles and testes

Male body mass was recorded at the start (week 1, day 1), middle (week 5) and end of the experiment (week 11). During the first four days of week 11, males were killed using an overdose of halothane. The order in which males were killed was random within treatments but balanced between treatments. Immediately after euthanasia, males were weighed (to the nearest 0.001 g) and measured using an electronic calliper (to the nearest 0.01 mm). Males were dissected and all paired preputial glands (hereafter called 'preputial gland'), seminal vesicles (hereafter called 'seminal vesicle'), testes (hereafter called 'testis') and epididymides (hereafter called 'epididymis') were weighed using an electronic balance (to the nearest 0.001 g). All masses and sperm measures (see below) were recorded blind to treatment group.

(e) Epididymal sperm count and sperm velocity measures

Immediately after dissection, the left epididymis was removed and the cauda was isolated in a Petri dish containing 150 µl of B.W.W. medium solution (Biggers Whitten Whittingham; see Bronson *et al.* 1989; Koyama & Kamimura 1999; **Chapter 4**). Using a scalpel blade, 10 incisions were performed in the cauda and the sperm were allowed to disperse for 1 min. Then, another 250 µl of B.W.W. was added. All 400 µl of B.W.W. was next transferred to an eppendorf tube and maintained in a water bath at 37°C while the right epididymis was dissected. To evaluate the number of sperm in the caput of the right epididymis, the same protocol as Ramm and Stockley (2009a) was followed. This tissue was placed in a Petri dish and the head and the body of the caput epididymis were macerated with a scalpel blade for 1 min in 1 ml of 1% citrate solution. Then, the preparation was mixed using a pipette and transferred to an Improved Neubaer haemocytometer, which was left to stand for 15 min in a sealed container on moist cotton wool. Finally, sperm counts were performed manually under a microscope using standard protocols (European Society of Human Reproduction and Embryology 2002). During the time the haemocytometer was left to stand, the sperm motility analysis was performed. Fifteen minutes after being added to the water bath, 10 µl of the solution contained in the eppendorf tube (see above) was put on a microscope slide and

covered with a cover slip. The slide was next placed under the microscope (Leica DM1000 Microscope) on a microstat heated stage (Brunel Microscopes) set up at 37°C. Several videos of sperm motility were then recorded during 2 seconds (75 frames per second, 150 frames in total for each video) at magnification X20 using a Pointgrey camera (FL2-03S2M-C), no more than 30 min after the start of the dissection.

Videos were analysed using Computer-Assisted Sperm Analysis (CASA) (Wilson-Leedy & Ingermann 2007), a Plugin implemented under ImageJ software (version 1.38x, http://rsbweb.nih.gov/ij/). Using this software, I first inverted the images, then adjusted the brightness and the contrast and finally adjusted and converted the threshold in order to select only the sperm heads for analysis. Several measurements were recorded: (1) curvilinear velocity (VCL, in μm/s) which estimates the velocity point to point along the trajectory (2) average path velocity (VAP, in μm/s) which estimates the point to point velocity over a constructed smooth path, and (3) straight line velocity (VSL, in μm/s) which estimates the velocity point to point along a straight line (**Figure 4.1**). The threshold values for excluding static sperm were set up as 25 μm/s for the VCL, 20 μm/s for the VAP and 3 μm/s for the VSL. The percentage of motile sperm (% motility) defined as the percent of sperm moving in a manner fitting motility determination parameters and the linearity (LIN = VSL/VAP) describing the path curvature were also calculated by the CASA Plugin (Wilson-Leedy & Ingermann 2007). For each individual, I recorded two videos. First, I analysed each video twice to test for a repeatability of the measures taken by CASA Plugin on the same video. These measures were highly repeatable for each variable (e.g. for VAP: intra-class coefficient of correlation: $r = 0.98$; $F_{53} = 115.73$; $P < 0.001$) and were therefore averaged. Next, I tested the repeatability of the measures between the two different videos recorded on the same individual using the average value of each video previously calculated. These measures were also highly repeatable (e.g. for VAP: intra-class coefficient of correlation: $r = 0.96$; $F_{27} = 51.29$; $P < 0.001$) and were therefore averaged to obtain a mean value of each sperm quality trait investigated.

(f) Estimates of daily sperm production

Daily sperm production rate was based on spermatid head counts from testicular homogenates (Amann & Lambiase 1969; Amann 1970). Because the timing of spermatogenesis in bank voles is known (Grocock & Clarke 1976), a static measure of sperm cells at the homogenization-resistant stage of spermatogenesis can be converted into a dynamic estimate of daily sperm production (sperm produced by the testis per day).

The same procedure as described by Seung *et al.* (2003) was followed. Frozen right testes were thawed for 1 min and the tunica albuginea was removed. Then, each testis was homogenized in 2 X 1 min stages in 10 ml dimethyl sulphoxide (DMSO)/saline solution using an Ystral X10/20 homogenizer with 10T shaft. Spermatids were then stained with Trypan blue, and spermatid heads were counted using an Improved Neubauer haemocytometer under 40 X magnification using standard protocols (European Society of Human Reproduction and Embryology 2002).

(g) Statistical analysis

Since the three descriptors of sperm velocity (VCL, VAP, VSL) were highly correlated (**Table 5.3**), a principal component analysis (PCA) of the variance-covariance matrix of these three log-transformed variables was performed to reduce the number of parameters in the following analyses. The first eigenvector factor extracted from the PCA summarized multivariate motility variation, explained 96.28% of the variance and had an eigenvalue of 2.88. The loadings of the three velocity measurements on this first factor were: 0.97 (VCL); 0.99 (VAP); 0.98 (VSL). The factor score is thus the single variable used in the subsequent analyses to represent sperm motility (hereafter called 'sperm motility factor').

The normality of the data was assessed using Kolmogorov-Smirnov tests and was improved when necessary by logarithmic transformations. All tests were two-tailed and were conducted using SPSS 16.0. Data are presented as means ± s.e.m and differences were regarded as statistically significant at $P < 0.05$.

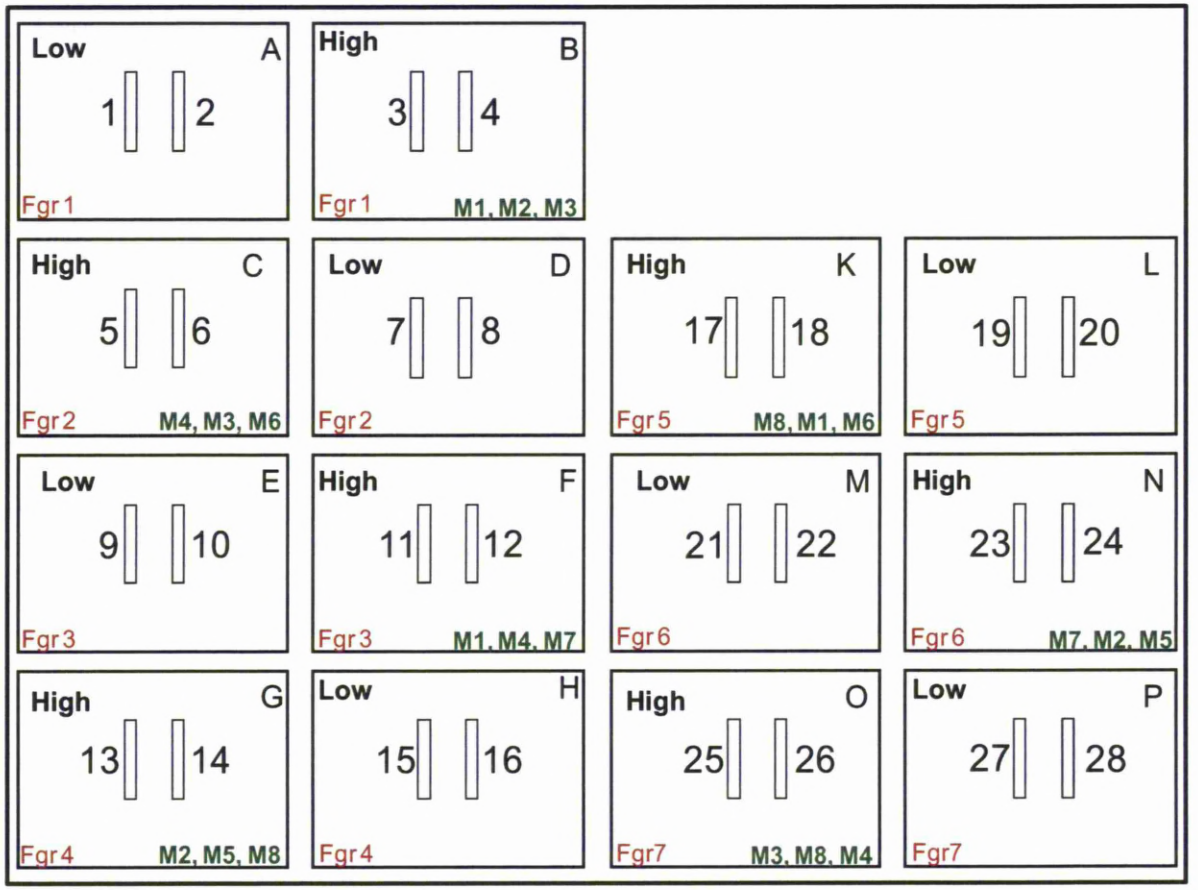

Figure 5.1 Experimental design and position of the cages in the 'enclosure room'. In each enclosure (1.2 m X 1.2 m) two M3 cages (see Methods) containing a single male were placed (n = 28), separated by 30 cm. Seven groups of three females (coded Fgr 1 to Fgr 7) and 8 males (M1 to M8) were used as external stimuli. On this Figure, the treatment group ('high' or 'low') and the code of the individuals used as external stimuli to provide social odours are indicated in each enclosure (A to P).

Table 5.1 Example of the experimental schedule for a subject in the 'high' competition group. Arbitrarily, I chose enclosure B (see Figure 5.1). Each Monday, cages were cleaned (except week 1 since the experiment started with clean cages); males were released sequentially in the enclosure for 1 hour, except weeks 2, 3 and 4 where they were released simultaneously for 30 mins. When males were housed back in the clean cages, an odour stimulus from the female was added. Each Tuesday, Wednesday and Friday bedding (12.5 g) from the males used as external stimuli was added and each Thursday 12.5 g of bedding were exchanged between the two males sharing the same enclosure (BEEM = Bedding Exchange Enclosure Males). Cages were cleaned on the last day to avoid measuring an immediate response to the last odour introduced in the cage.

High competition group					
Week	Monday	Tuesday	Wednesday	Thursday	Friday
1	Fgr 1	M1	M2	BEEM	M3
2	Cage cleaning + Direct Contact + Fgr 1	M1	M2	BEEM	M3
3	Cage cleaning + Direct Contact + Fgr 1	M1	M2	BEEM	M3
4	Cage cleaning + Direct Contact + Fgr 1	M1	M2	BEEM	M3
5	Cage cleaning + Direct Contact + Fgr 1	M1	M2	BEEM	M3
6	Cage cleaning + Direct Contact + Fgr 1	M1	M2	BEEM	M3
7	Cage cleaning + Direct Contact + Fgr 1	M1	M2	BEEM	M3
8	Cage cleaning + Direct Contact + Fgr 1	M1	M2	BEEM	M3
9	Cage cleaning + Direct Contact + Fgr 1	M1	M2	BEEM	M3
10	Cage cleaning + Direct Contact + Fgr 1	M1	M2	BEEM	M3 +Cage cleaning

Table 5.2 Example of the experimental schedule for a subject in the 'low' competition group. Arbitrarily, I chose enclosure A (see Figure 5.1). Each Monday, cages were cleaned (except week 1 since the experiment started with clean cages); males were released sequentially in the enclosure for 1 hour, except weeks 2, 3 and 4 where they were released simultaneously for 30 mins. When males were housed back in clean cages, an odour stimulus from the female was added (12.5 g of bedding). Each Tuesday, Wednesday and Friday 12.5g of clean bedding were added (CB) and each Thursday 12.5g of bedding were exchanged between the two males sharing the same enclosure (Bedding Exchange Enclosure Males = BEEM). Cages were cleaned on the last day to avoid measuring an immediate response to the last odour introduced in the cage.

Low sperm competition group					
Week	**Monday**	**Tuesday**	**Wednesday**	**Thursday**	**Friday**
1	Fgr 1	CB	CB	BEEM	CB
2	Cage cleaning + Direct Contact + Fgr 1	CB	CB	BEEM	CB
3	Cage cleaning + Direct Contact + Fgr 1	CB	CB	BEEM	CB
4	Cage cleaning + Direct Contact + Fgr 1	CB	CB	BEEM	CB
5	Cage cleaning + Direct Contact + Fgr 1	CB	CB	BEEM	CB
6	Cage cleaning + Direct Contact + Fgr 1	CB	CB	BEEM	CB
7	Cage cleaning + Direct Contact + Fgr 1	CB	CB	BEEM	CB
8	Cage cleaning + Direct Contact + Fgr 1	CB	CB	BEEM	CB
9	Cage cleaning + Direct Contact + Fgr 1	CB	CB	BEEM	CB
10	Cage cleaning + Direct Contact + Fgr 1	CB	CB	BEEM	Cage cleaning

Table 5.3 Correlation matrix of sperm velocity traits (Pearson coefficients). All correlations are based on $n = 28$. Each variable has been log-transformed prior to correlations. All correlations are significant at $P < 0.001$ (VAP: velocity average path; VCL: velocity curvilinear; VSL: velocity straight line).

	VAP	VCL	VSL
VAP		0.951 (<0.001)	0.986 (<0.001)
VCL			0.895 (<0.001)
VSL			

5.4 Results

(a) Effect of social experience

At the end of the experiment (week 11), no difference was found in the body mass of subjects in 'low' and 'high' competition groups (low: $\overline{X} = 22.91 \pm 0.66$ g, high: $\overline{X} = 23.02 \pm 0.63$ g; $t_{26} = -0.14$, $P = 0.89$) (**Figure 5.2**). Similarly, there was no difference in the body mass of males in the two treatment groups at the start of the experiment (week 1; see methods) or in the middle (week 5) (low: $\overline{X} = 22.17 \pm 0.69$ g, high: $\overline{X} = 21.66 \pm 0.60$ g; $t_{26} = 0.51$, $P = 0.62$). Final and intermediate body masses were highly correlated ($r = 0.93$, $n = 28$, $P < 0.001$) but were not correlated with the body mass at the start of the experiment (not shown). A repeated-measures ANOVA incorporating the three body masses (start, intermediate, end) revealed no effect of treatment group ('low' versus 'high' competition group) ($F_{2,26} = 0.29$, $P = 0.75$). Therefore, male's growth was not influenced by the experimental treatment.

Males from the two groups did not differ significantly in their sperm production rate. There was no difference in the epididymal sperm count (low: $\overline{X} = 5.61 \pm 0.88 \times 10^6$, high: $\overline{X} = 6.06 \pm 0.67 \times 10^6$, $t_{26} = -0.72$, $P = 0.48$) or in the number of sperm produced daily (low: $\overline{X} = 3.35 \pm 0.95 \times 10^6$, high: $\overline{X} = 3.00 \pm 0.61 \times 10^6$; $t_{26} = 0.78$, $P = 0.44$) (**Figure 5.3**). The 'sperm motility factor' was not significantly different between males from the two treatment groups ($t_{26} = 0.09$, $P = 0.93$).

Similarly, males from the two treatment groups did not differ significantly in their testis mass (low: $\overline{X} = 0.57 \pm 0.02$ g, high: $\overline{X} = 0.57 \pm 0.01$ g; $F_{1,27} = 0.01$, $P = 0.90$) or epididymis mass (low: $\overline{X} = 0.13 \pm 0.004$ g, high: $\overline{X} = 0.14 \pm 0.003$ g; $F_{1,27} = 3.65$, $P = 0.07$) (**Figure 5.4**). However, males from the 'high' competition group had significantly heavier seminal vesicles than males from the 'low' competition group (low: $\overline{X} = 0.13 \pm 0.009$ g, high: $\overline{X} = 0.16 \pm 0.007$ g; $F_{1,27} = 5.60$, $P = 0.03$) (**Figure 5.4**). All these results were obtained with a general linear model with the organ mass as the dependent variable, treatment group as a fixed factor and body mass as a covariate, but were quantitatively unchanged when the organ mass were compared by independent t-tests based on enclosure means (**Table 5.4**).

Table 5.4 Differences in traits investigated between males from the 'low' and 'high' competition groups based on enclosure means. $*P < 0.05$

	Group	mean	s.e.m	d.f	*t*	*P*
Body mass (g)						
	Low	22.91	0.33	12	0.13	0.90
	High	23.02	0.56	12		
Testis mass (g)						
	Low	0.57	0.03	12	0.26	0.80
	High	0.57	0.01	12		
Epididymis mass (g)						
	Low	0.13	0.004	12	1.89	0.08
	High	0.14	0.003	12		
Seminal vesicle mass (g)						
	Low	0.13	0.01	12	2.28	0.02 *
	High	0.16	0.01	12		
Epididymal sperm count ($x10^6$)						
	Low	5.61	1.00	12	0.86	0.40
	High	6.06	0.32	12		
Daily sperm production ($x10^6$)						
	Low	3.35	0.38	12	-0.65	0.53
	High	3.00	0.16	12		

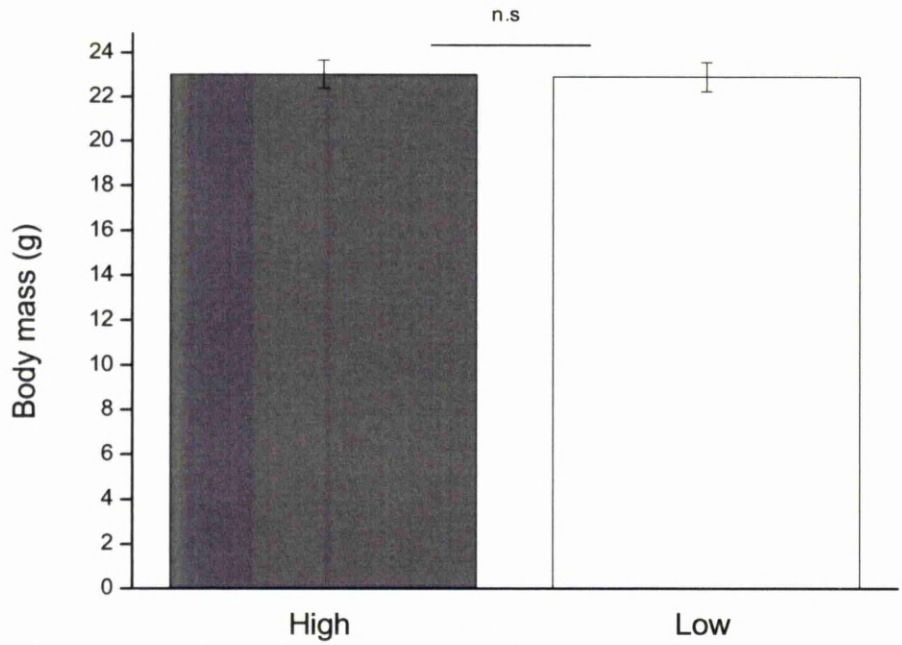

Figure 5.2 Body mass for males from the 'high' and 'low' competition groups. Males from these two groups do not differ significantly in their body mass at the end of the experiment (t_{26} = -0.14, P = 0.89). Bars represent mean ± s.e.m.

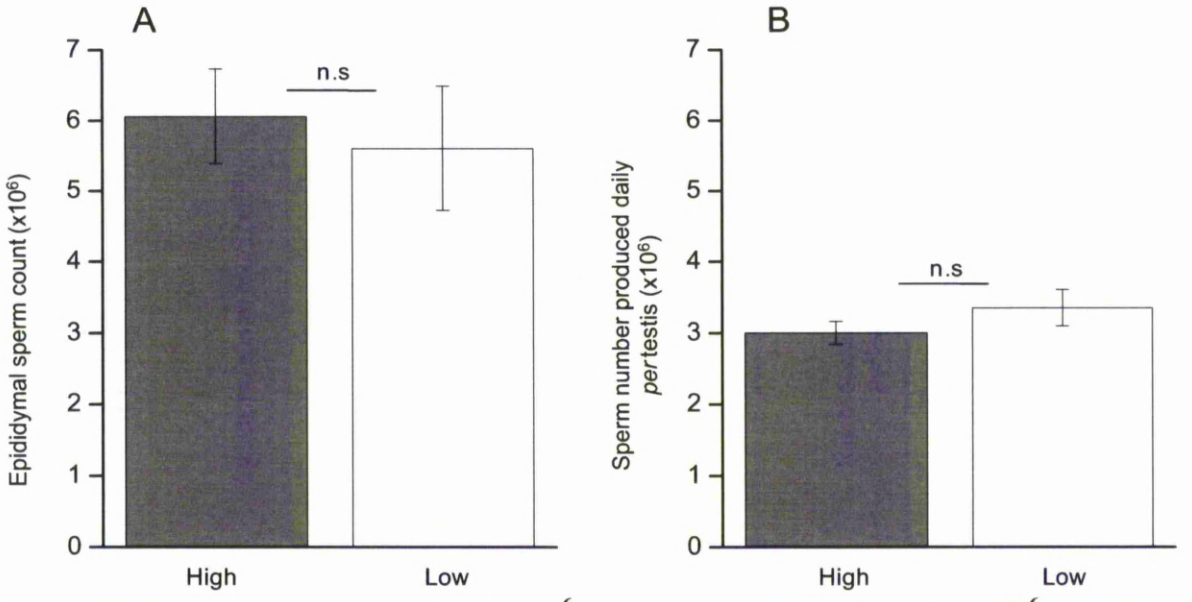

Figure 5.3 Epididymal sperm count (x10^6) (A) and number of sperm (x10^6) produced daily per testis (B) for males from the 'high' and 'low' competition groups. In both cases differences between males from the two competition groups are not significant (Epididymal sperm count: t_{26} = -0.72, P = 0.48 – Number of sperm produced daily: t_{26} = 0.78, P = 0.44). Bars represent mean ± s.e.m.

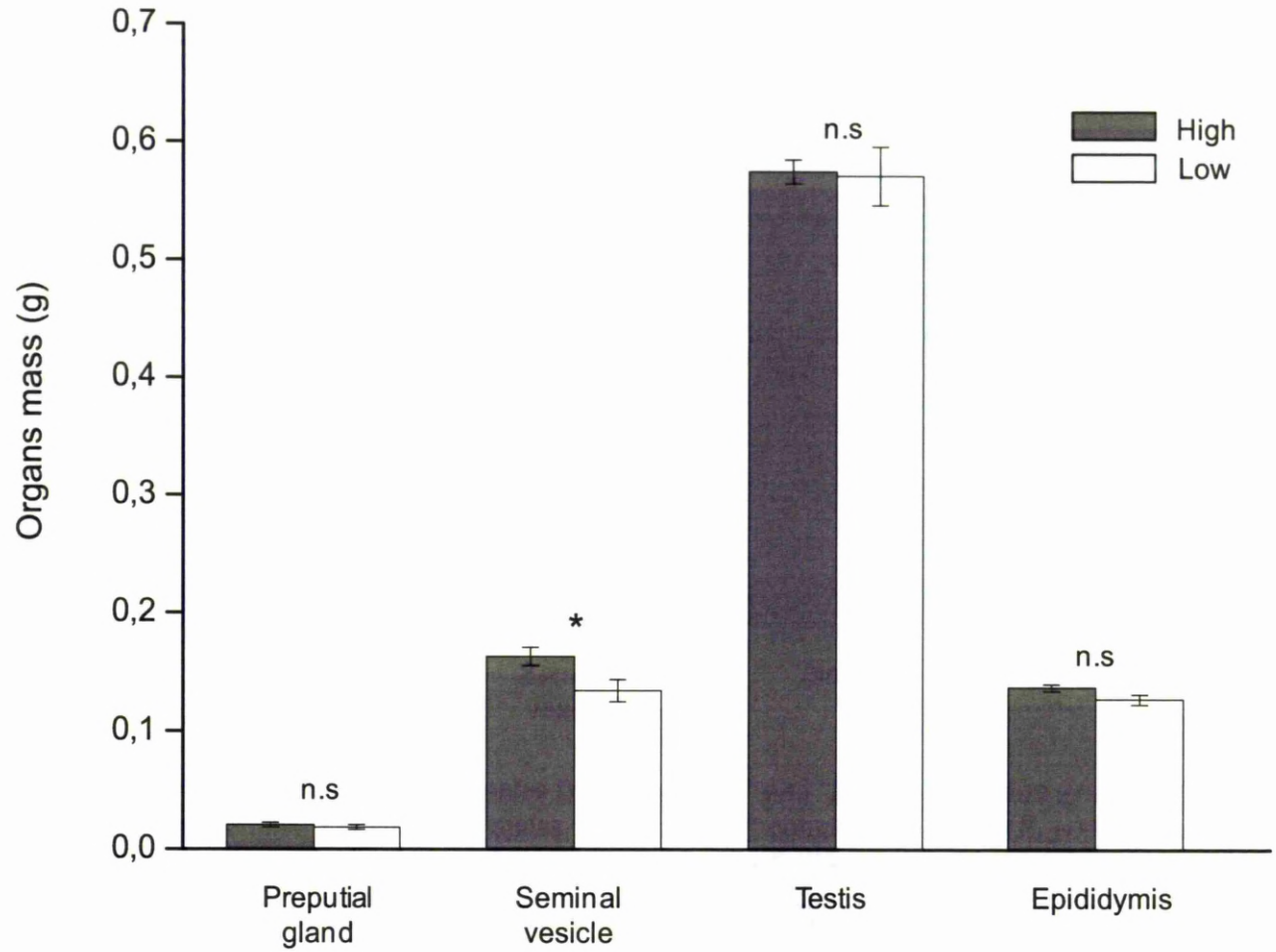

Figure 5.4 Mass of different reproductive traits for males from the 'high' and 'low' competition groups. Males from the 'high' competition group have significantly heavier seminal vesicles than males from the 'low' competition group ($F_{1,27} = 5.60$, $P = 0.03$). Preputial gland mass, testis mass and epididymis mass did not significantly differ between males for the two competition groups (Preputial gland: $F_{1,27} = 0.45$, $P = 0.51$ – Testis mass: $F_{1,27} = 0.01$, $P = 0.90$ – Epididymis mass: $F_{1,27} = 3.65$, $P = 0.07$). Bars represent mean ± s.e.m.

(b) Sperm production and investment in scent marking

The rationale for having two males in each enclosure was to obtain clearly different patterns of scent marks. However, only pairs of males from 5 of 14 enclosures (3 in the 'low' competition group and 2 in the 'high' competition group) showed distinctly different patterns of scent marks, and variation in the number of scent marks between males was relatively large (Range: 5.66 - 70.33; $\overline{X} = 27.19 \pm 3.53$). Males from the two treatments did not significantly differ in the number of scent marks produced (low: $\overline{X} = 31.26 \pm 4.96$, high: $\overline{X} = 23.12 \pm 4.96$; $t_{26} = 1.16$, $P = 0.26$) or in their preputial gland mass (as shown in **Section 5.4.a**), which suggests that the proportion of males investing strongly in pre-copulatory competition did not differ between the two groups. No correlation was found between the number of scent marks and the preputial gland mass ($r = -0.06$, $n = 28$, $P = 0.77$).

Overall, the available data do not support the idea of a trade-off between investment in scent marking and sperm production. No negative correlations were found between male investment in scent marking (i.e. number of scent marks) and any of the traits related to sperm production effort (epididymal sperm count: $r = 0.08$, $n = 28$, $P = 0.68$; number of sperm produced daily: $r = -0.07$, $n = 28$, $P = 0.71$; testis mass: $r = 0.27$, $n = 28$, $P = 0.17$; epididymis mass: $r = 0.17$, $n = 28$, $P = 0.38$) or sperm motility ($r = -0.18$, $n = 28$, $P = 0.35$).

Finally, I tested for an interaction between investment in scent marking and 'competition group', to explain traits related to sperm production, using a GLM including the competition group ('low' versus 'high') as a fixed factor and the scent marking investment as a covariate. The interaction between 'competition group' and 'scent marking investment' significantly explained the testis mass ($F_{1,27} = 6.86$, $P = 0.02$) and the epididymis mass ($F_{1,27} = 6.11$, $P = 0.02$) but not the epididymal sperm count ($F_{1,27} = 1.20$, $P = 0.28$), the number of sperm produced daily ($F_{1,27} = 0.91$, $P = 0.35$) or the sperm motility ($F_{1,27} = 2.43$, $P = 0.13$) (**Table 5.5**). This significant interaction comes from a significant positive correlation between scent marking rate and testis mass and between scent marking rate and epididymis mass in the 'low' competition group (**Figure 5.5**; **Figure 5.6**), whereas these variables were not correlated in the 'high' competition group (low: testis mass: $r = 0.54$, $n = 14$, $P = 0.04$,

epididymis mass: $r = 0.58$, $n = 14$, $P = 0.03$ – high: testis mass: $r = -0.37$, $n = 14$, $P = 0.19$, epididymis mass: $r = -0.26$, $n = 14$, $P = 0.37$). However, if in the 'low' competition group scent marking is significantly positively correlated with both the testis mass and the epididymis, that is due to the strong correlation between these two organs ($r = 0.77$, $n = 14$, $P = 0.001$).

Table 5.5 Analysis of testis mass, epididymis mass, epididymal sperm count and number of sperm produced in a GLM including the treatment group ('high' versus 'low') as a factor and the investment in scent marking as a covariate. Analyses were conducted on log-transformed data. * = $P < 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

Variables		Mean square	$F_{1,27}$	P
Testis mass				
	Group	0.018	6.86	0.02*
	Scent mark	0.005	1.92	0.18
	Interaction group x scent mark	0.017	6.31	0.02*
Epididymis mass				
	Group	0.015	8.81	0.01**
	Scent mark	0.003	1.67	0.21
	Interaction group x scent mark	0.010	6.11	0.02*
Epididymal sperm count				
	Group	0.098	1.57	0.22
	Scent mark	0.013	0.22	0.65
	Interaction group x scent mark	0.075	1.20	0.28
Number of sperm produced daily				
	Group	0.008	0.54	0.47
	Scent mark	0.006	0.43	0.52
	Interaction group x scent mark	0.013	0.91	0.35
Sperm motility				
	Group	2.360	2.39	0.13
	Scent mark	0.616	0.62	0.44
	Interaction group x scent mark	2.397	2.43	0.13

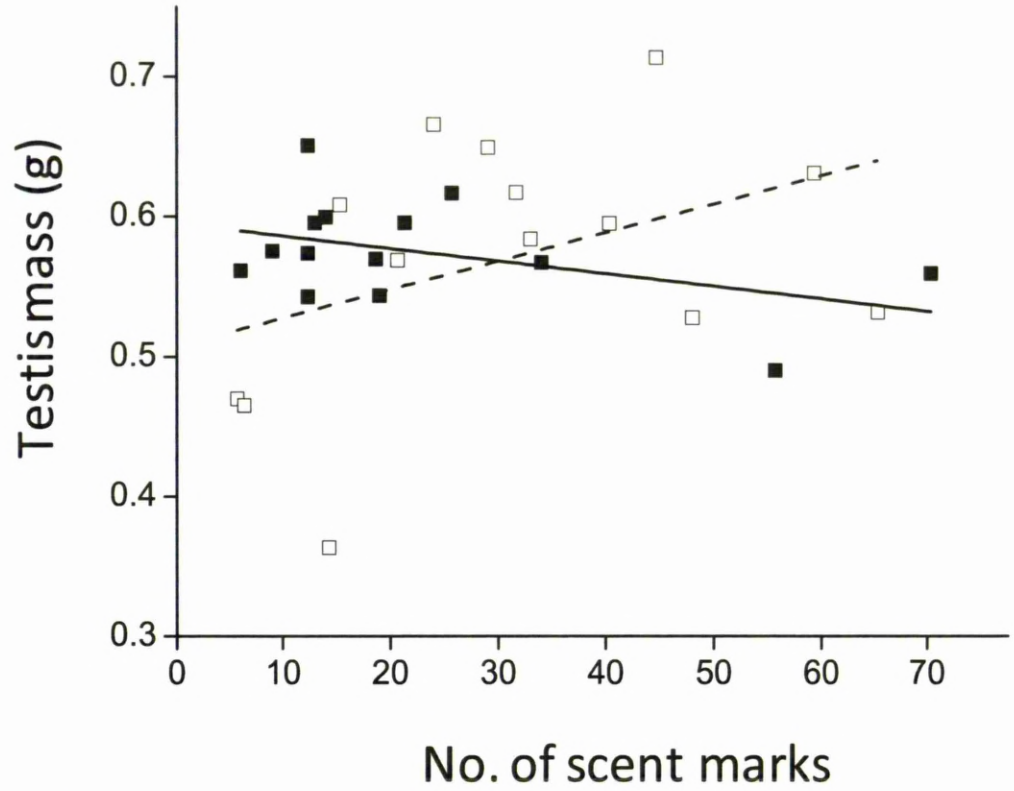

Figure 5.5 Relationship between testis mass and the average investment in scent marks for males from the 'low' competition group (open squares, dash line) and males from the 'high' competition group (filled squares, black line). There is a significant positive relationship between testis mass and scent mark investment for males from the 'low' competition group (r = 0.54, n = 14, P = 0.04) but not for males from the 'high' competition group (r = -0.37, n = 14, P = 0.19). Overall, the interaction between scent marking investment and the 'group' effect is significant ($F_{1.27}$ = 6.61, P = 0.02).

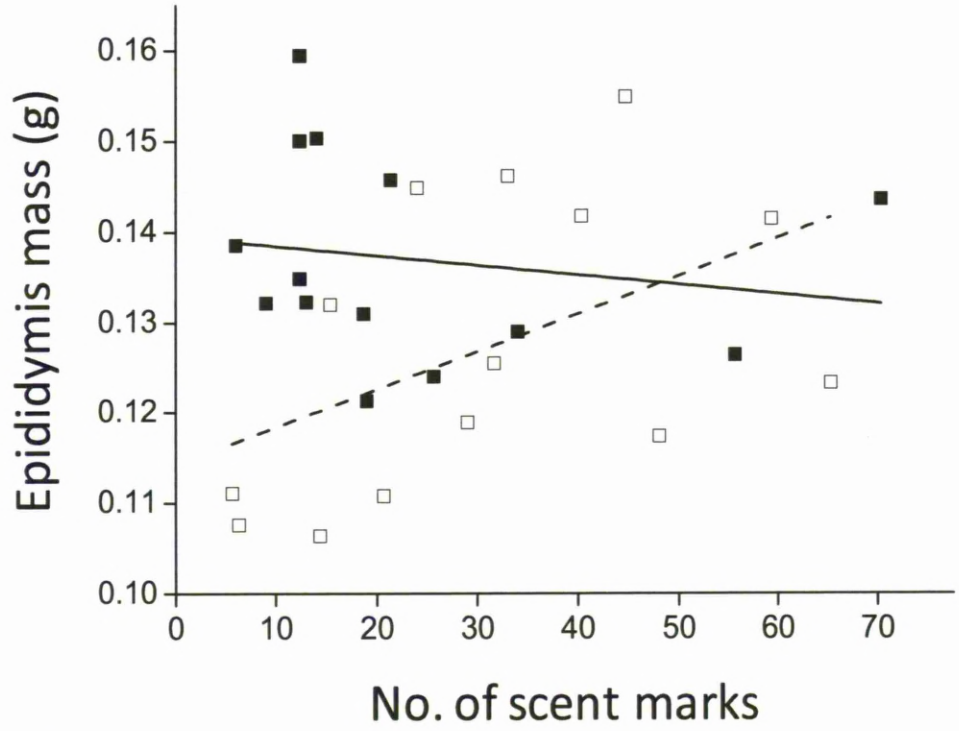

Figure 5.6 Relationship between epididymis mass and the average investment in scent marks for males from the 'low' competition group (open squares, dash line) and males from the 'high' competition group (filled squares, black line). There is a significant positive relationship between epididymis mass and scent mark investment for males from the 'low' social competition group ($r = 0.59$, $n = 14$, $P = 0.03$) but not for males from the 'high' competition group ($r = -0.26$, $n = 14$, $P = 0.37$). Overall, the interaction between scent marking investment and the 'group' effect is significant ($F_{1,27} = 6.11$, $P = 0.02$).

5.5 Discussion

(a) Ejaculate production according to social experience

The first important result revealed by this study is that male bank voles did not increase investment in sperm production according to social cues of average sperm competition level. Individuals from the 'low' and the 'high' competition group did not significantly differ in their number of sperm in the caput epididymis, in their daily sperm production or in sperm motility. These findings contrast with the pattern found in house mice (Ramm & Stockley 2009a). I also found no difference in testis mass between the two treatment groups.

A possible explanation of the absence of response in bank vole reproductive investment could be that the odours used to induce perception of different sperm competition levels are not the stimuli used by male bank voles to assess their social environment. However, this seems unlikely since olfactory communication is the key system used by rodents in social and sexual communication (delBarco-Trillo & Ferkin 2004; Hurst & Beynon 2004; Villavicencio *et al.* 2009) including bank voles (Kapusta *et al.* 1996; Marchlewska-Koj 2000; Radwan *et al.* 2008).

With a highly comparable protocol, Ramm and Stockley (2009a) found in house mice that sperm production increases with the level of competition (their 'low' competition group corresponds to the presence of one competitor and their 'high' competition group corresponds to three competitors, whereas it is four in the present study). Therefore, bank voles and house mice appear to differ in their response to social cues of average sperm competition level and this difference might be explained by their social organisation and mating system. House mice are polygynous but females seek extraterritorial copulations (Bronson 1979; Dean *et al.* 2006). In this species, the proportion of multiple sired litters is 20% but increases in high density populations (Dean *et al.* 2006) whereas in bank voles, a promiscuous species, the proportion of multiply sired litters is 35.5% in the wild (Ratkiewicz & Borkowska 2000) and 92% of females initiate a second mating, at least under laboratory conditions (Klemme *et al.*

2006a). Therefore, the risk of sperm competition is higher in bank voles, which is confirmed by their adaptation of larger relative testis size (Kenagy & Trombulak 1986; Ramm *et al.* 2005). Since bank voles are adapted to a high risk of sperm competition which is relatively constant across populations (Klemme *et al.* 2006a; Kruczek & Zatorska 2008), it is likely that their basal investment in sperm production is fixed and relatively high. Therefore, there are perhaps less opportunities for further increases in sperm production via developmental plasticity and/or phenotypic flexibility (Piersma & Drent 2003; Auld *et al.* 2010). Moreover, potential phenotypic plasticity allowing for example individuals from the 'low' competition group to reduce investment in sperm production might be counter-selected by the likelihood of environmental variation (i.e. a return to high levels of competition) (deWitt *et al.* 1998). These results, in contrast with the pattern found in mice, emphasize the importance of studying species with different social organizations in order to understand the costs (*sensu* phenotype-environment mismatching; Auld *et al.* 2010) and limits of phenotypic plasticity (deWitt *et al.* 1998; Auld *et al.* 2010) acting on spermatogenesis.

Male bank voles that had experienced a 'high' level of competition had heavier seminal vesicles compared to those that experienced a 'low' level of competition. Assuming that, similar to other species (e.g. *D. melanogaster*, Wigby *et al.* 2009), males with larger seminal vesicles will produce more seminal fluid proteins, our findings suggest that male bank voles respond to the average risk of sperm competition through an adjustment of the quantity of these proteins produced. In mammals, functions of seminal fluid are various (review in Clavert *et al.* 1990; Poiani 2006) such as the production of copulatory plugs in rodents and primates (Clavert *et al.* 1990; Poiani 2006; Dixson 1998). If we consider that males who experience a high level of sperm competition invest substantially in plug production, it suggests that an average risk of sperm competition in the population might select for a 'defensive' strategy (copulatory plug investment) rather than an 'offensive' strategy (sperm production investment). However, bank vole males generally remove successfully the copulatory plug of a previous male and the presence of a plug does not appear to inhibit the female's propensity to remate (Klemme *et al.* 2006a; Klemme *et al.* 2007a), suggesting that an increase of seminal fluid production when the level of sperm competition is 'high' might benefit males through another function of these proteins. For instance, seminal fluid proteins can enhance sperm motility (Chapman 2001; Poiani 2006; but see **Chapter 4**), mating rate (Crudgington *et al.* 2009) and can decrease the fertilization efficiency of rival sperm (i.e.

allospermicide function, see Poiani 2006). In insects, seminal fluid proteins play a critical role in sperm competition (Chapman & Davies 2004; Fricke *et al.* 2009; Wigby *et al.* 2009; Bretman *et al.* 2010) but such roles in mammals are still poorly investigated. Our results suggest than in bank voles and probably in other mammalian species, the role of seminal fluid in sperm competition might have been underestimated, especially since recent comparative studies revealed that: i) in rodents the size of the seminal vesicle is correlated with the intensity of sperm competition (Ramm *et al.* 2005) and ii) the divergence rate of a seminal fluid protein (SVS II), involved in the copulatory plug formation, is favoured under high levels of sperm competition (Ramm *et al.* 2009). Further experiments are now necessary to understand the function of seminal fluid proteins in mammals in the context of post-copulatory competition.

(b) Sperm production and scent marking investment

The absence of significant differences in preputial gland mass and scent marking rate between individuals from the 'low' and 'high' competition groups suggests that the treatment did not influence the likelihood for a male to invest in pre-copulatory competition. In many rodents such as bank voles, males use scent marks to communicate their social status and to attract females (Rozenfeld *et al.* 1987; Roberts 2007). This investment in sexual signalling through preputial glands is costly (Radwan *et al.* 2006; **Chapter 4**), but surprisingly, I did not find a correlation here between scent marking rate and preputial gland mass.

In bank voles, dominant males invest more in scent marking compared to subordinates (Rozenfeld *et al.* 1987; **Chapter 4**) and females prefer mating with dominant males (Horne & Ylönen 1996; Kruczeck 1997). Since males have to tailor the quantity of resources allocated to reproduction between investment in gaining mating and investment in sperm production/quality (Parker 1998), males that invest substantially in scent marking (i.e. gaining mating) are predicted to decrease their investment in sperm production and sperm quality (Parker 1990a; Tazzyman *et al.* 2009). However, I found no evidence of a negative relationship between investment in scent marking and sperm production (i.e. epididymal sperm count and number of sperm produced daily). In contrast, a negative relationship between epidydimal sperm count and scent marking rate was found for house mice in 'low' and in 'high' competition groups (Ramm & Stockley 2009a). Moreover, I found no

relationship between investment in scent marking and sperm quality (assessed through sperm motility).

When analysis was restricted to individuals of the 'low' competition group, I found a positive correlation between the scent marking rate and the mass of both the testis and epididymis, which suggests that in this group, males who invest more in pre-copulatory competition are likely to invest substantially more in organs involved in post-copulatory competition (i.e. testis, epididymis). Why no association between scent marking behaviour and organs related to male fertility was found in the 'high' competition group remains unclear. Overall then, my results suggest that male bank voles do not trade-off between their investment in social status and their investment in sperm production. However, the absence of trade-off between traits involved in pre- and post-copulatory sexual selection is not uncommon. For example, in the red deer (*Cervus elaphus*), antler size is positively associated with relative testis size and sperm motility (Malo *et al.* 2005b; see also Locatello *et al.* 2006; **Chapter 4**).

(c) Conclusion

I found no evidence of plasticity in sperm production and quality for male bank voles according to the level of social experience with other males. Since this contrasts with findings for house mice, it underlines the importance of studying different species in order to understand phenotypic plasticity in sperm investment (Auld *et al.* 2010). Moreover, scent marking investment had no effect on sperm production / quality and my results do not support the idea of a general trade-off between investment in pre- and post-copulatory traits, at least in bank voles. Interestingly, males who experienced a high level of sperm competition had larger seminal vesicles, suggesting that seminal fluid proteins may play an important role in sperm competition or in promoting an increasing mating rate in this species.

Adaptations to immediate sperm competition risk or intensity have been well described in mammals in the last decade (delBarco-Trillo & Ferkin 2004; Pound & Gage 2004; Kilgallon & Simmons 2005; delBarco-Trillo & Ferkin 2006; Ramm & Stockley 2007) but the influence

of longer term social experience (see Engqvist & Reinhold 2005) on ejaculate investment is less well studied. Future experiments should investigate how males reared at different levels of social experience tailor their ejaculate in response to the immediate risk or intensity of sperm competition (Parker *et al.* 1996; Parker *et al.* 1997; Engqvist & Reinhold 2005). However, it will also be important to tease apart the investment in sperm and seminal fluid proteins in these experiments (see also Cameron *et al.* 2007).

Chapter 6: Sperm competition and brain size evolution in mammals[1]

6.1 Chapter overview

The 'expensive tissue hypothesis' predicts a size trade-off between the brain and other energetically costly organs. A specific version of this hypothesis, the 'expensive sexual tissue hypothesis', argues that selection for larger testes under sperm competition constrains brain size evolution. I show here that there is no general evolutionary trade-off between brain and testis mass in mammals. The predicted negative relationship between these traits is not found for rodents, ungulates, primates, carnivores, or across combined mammalian orders, and neither does total brain mass vary according to the level of sperm competition as determined by mating system classifications. Although I am able to confirm previous reports of a negative relationship between brain and testis mass in echolocating bats, the results suggest that mating system may be a better predictor of brain size in this group. I conclude that the expensive sexual tissue hypothesis accounts for little or none of the variance in brain size in mammals, and suggest that a broader framework is required to understand the costs of brain size evolution and how these are met.

6.2 Introduction

Understanding the factors that influence brain size evolution is a subject of longstanding interest within evolutionary biology research, in terms of both structural and functional considerations (Finlay & Darlington 1995; Barton & Harvey 2000; Barton 2006; Byrne & Bates 2007; Dunbar & Shultz 2007; Sol *et al.* 2007; Barrickman *et al.* 2008; Gonzalez-Voyer *et al.* 2009; Isler & van Schaik 2009). In particular, several, not necessarily incompatible, hypotheses have been proposed to explain selection for relatively large brain size in certain birds and mammals, including ecological hypotheses (Harvey *et al.* 1980; Barton 1998; Sol *et al.* 2005), and the social brain hypothesis (Brothers 1990; Dunbar 1998). Other hypotheses focus on potential evolutionary constraints on encephalization in these groups; for example

[1] This chapter is modified from: Lemaître, J.-F., Ramm, S., Barton, R. A. & Stockley, P. 2009. Sperm competition and brain size evolution in mammals. *Journal of Evolutionary Biology*, 22, 2215-2221.

the 'expensive tissue hypothesis' of Aiello & Wheeler (1995) proposes that brain size is constrained by an evolutionary trade-off between investment in energetically expensive brain tissue and other costly organs (e.g. the gut in primates).

More recently, attention has focussed on the possible role of sexual selection in brain size evolution among birds and mammals (Madden 2001; Garamszegi *et al.* 2005a; Lindenfors *et al.* 2007). In this context, Pitnick *et al.* (2006) suggested a new 'expensive sexual tissue hypothesis', whereby investment in brain tissue is proposed to trade-off specifically with investment in costly sexually selected traits such as large testes favoured under sperm competition. Sperm competition is a widespread phenomenon where sperm from two or more males compete to fertilize a set of ova (Parker 1970; Birkhead & Møller 1998), and is an important driving force in the evolution of diverse male reproductive traits that influence differential fertilization success, from sperm morphology and ejaculate characteristics to copulatory and mate-guarding behaviour (Birkhead & Møller 1998). In mammals, it is well established that average testis mass (relative to body mass) is positively correlated with the level of sperm competition typically experienced by males (e.g. ungulates, Ginsberg & Rubenstein 1990; primates, Harcourt *et al.* 1981, 1995; bats, Hosken 1997, 1998; rodents, Ramm *et al.* 2005). This is because larger testes allow males to invest more sperm in each reproductive event, thereby increasing the probability of winning in sperm competition (Parker *et al.* 1997; Wedell *et al.* 2002; see also Preston *et al.* 2003; Schulte-Hostedde & Millar 2004; **Chapter 4**).

To date, support for the 'expensive sexual tissue hypothesis' has only been presented for bats, among which Pitnick *et al.* (2006) reported evidence of a negative evolutionary relationship between brain size and testis size as well as a larger brain (relative to body size) for monogamous or polygynous species compared to polygynandrous species (but see Dechmann & Safi 2009). However, there is more widespread evidence in other mammalian taxa to suggest an association between mating system and brain size: monogamous species generally have larger brains than do species where females mate multiply (primates, Schillaci 2006; carnivores and ungulates, Shultz & Dunbar 2007), suggesting that large brain size is often found in species with relatively low levels of sperm competition. Whereas Pitnick *et al.* (2006) argued that various bat species have evolved greater levels of investment in either brain or testis mass, but not both because each of these tissues is energetically expensive to

produce (c.f. Aiello & Wheeler 1995), Shultz & Dunbar (2007) suggest instead that larger brains might be an advantage for monogamous species to optimize mate choice and to avoid cuckoldry. These different interpretations emphasize the importance of considering whether or not correlations are robust to potentially confounding variables and analysis across broader comparative datasets (Barton 2006; Healy & Rowe 2007).

Here, I test for evidence of a widespread influence of sperm competition on brain size evolution across a broad taxonomic spectrum of mammals. I use a large dataset, comprising rodents, bats, ungulates, primates, carnivores and insectivores, to look for evidence of a negative relationship between brain size and testis size, as predicted by the expensive sexual tissue hypothesis (Pitnick *et al.* 2006). Results of these analyses do not support predictions of the expensive sexual tissue hypothesis, and suggest that it does not provide a general explanation for mammalian brain size evolution.

6.3 Methods

(a) Dataset

I collected data on total brain mass, testis mass, body mass and mating system. When data for continuous variables were available from multiple sources in the literature for the same biological trait in the same species, I used the mean of these data weighted by the sample size provided by each study. Because the different data sources often do not provide information on the sex of each animal measured, previous studies, including Pitnick *et al.* (2006), have used combined measures. I have therefore, followed the same procedure here, such that brain and body masses should be considered as an average size for males and females. All data are provided in the **Appendix**.

Data on total brain masses come principally from published reviews (e.g. see Mace *et al.* (1981) and McNab & Eisenberg (1989) for rodents and insectivores, Pitnick *et al.* (2006) for bats, Harvey *et al.* (1980) for primates, Shultz & Dunbar (2006) for ungulates, and Gittleman (1986) for carnivores). This dataset was then supplemented using primary sources identified from *Zoological Record* and the *Mammalian Species* series (see **Appendix** for a complete reference list).

For testis mass, the data utilised in this study come mainly from published reviews (e.g. Kenagy & Trombulak (1986) for rodents, insectivores and carnivores; Pitnick *et al.* (2006) for bats; Harcourt *et al.* (1981) for primates; and Ginsberg & Rubenstein (1990) for ungulates). Again, this dataset was supplemented using information from primary sources identified from *Zoological Record* and the *Mammalian Species* series (see **Appendix**). For each species, combined testis mass (hereafter testis mass) is the average mass of both (right and left) testes, measured from multiple adult males in reproductive condition.

For each species, I attempted to utilise body mass data obtained from the same study as brain mass. When this source did not provide information on body mass, I used information from testis mass sources to complete our dataset. In bats, body mass values are the same as those collated by Pitnick *et al.* (2006) and summarized in their electronic appendix. However, these authors provide two body masses for each species, one based on the source from where they obtained brain mass data and one from the source where they obtained testis mass data. Since these two body masses were strongly correlated ($r = 0.96$; $P < 0.001$) and because there was no significant difference between them for each species ($t_{74} = -1.61$, $P = 0.11$), I here present results only for analyses using the body mass data which originates from the same sources as the brain mass data. This choice is more appropriate for our analysis since brain mass is always included as the dependent variable in all tests of the expensive sexual tissue hypothesis. Unless otherwise stated, all of these results remain qualitatively unchanged if the alternative body mass measure is used instead.

For mating system, I employed a dichotomous classification to distinguish between species that are likely to experience relatively high versus relatively low levels of sperm competition. My classification was based on the number of males with which a female typically mates within a single reproductive bout. Thus, species in which females typically mate with more than one male per reproductive attempt were classified as 'high sperm competition' (including species with polyandrous, polygynandrous, and promiscuous mating systems) and those in which females typically mate with only one male per reproductive attempt were classified as 'low sperm competition' (including species with polygynous and monogamous mating systems). This classification allows me to test the hypothesis of an evolutionary correlation between level of sperm competition and brain mass across all mammals in the dataset for

which mating system data were collated (165 species) in a single model. Sources used to identify the level of sperm competition of each species are provided in the **Appendix**.

(b) Comparative methods

Comparisons between traits at the inter-specific level have a long history in evolutionary and behavioural ecology (Harvey & Pagel 1991) and recent development in statistical analysis have provided powerful tools to test for correlations between biological or behavioural traits across taxa (Felsenstein 1985; Harvey & Pagel 1991; Purvis & Rambaut 1995; Felsenstein 2008). In these analyses, confounding effects can easily create or mask significant correlations. To minimize such errors in comparative studies, it is advocated to take into account the effects of both body size scaling (allometry) and phylogenetic inertia on the traits to be analysed (Harvey & Pagel 1991; Cézilly *et al.* 2008).

Effects of body size are relatively straightforward to remove. The relationship between brain mass and body mass is non-linear (note that this is not specific to the brain, and applies to the majority of organs) and takes the form of an allometric relationship ($Y = aX^b$; see also **Chapter 3**). Thus when both brain mass and body mass are log-transformed the relationship becomes $\log(Y) = (b)\log(X) + \log(a)$ and one can seek the scaling component b as the slope of the linear relationship between log brain mass and log body mass. An efficient method to remove the effect of body mass is to include the log-value of body mass as a covariate in linear regression models used to explain the log-value of brain mass (i.e. dependent variable) (Havey & Pagel 1991; see also Freckleton 2009).

The second methodological issue in comparative studies is phylogenetic inertia (Harvey & Pagel 1991). To be compared in conventional statistical analyses, species need to be independent. However, closely related species share a substantial degree of common ancestry and are therefore more likely to share similar traits compared to species that diverged a long time ago (Felsenstein 1985). Absence of correction for this non-independance of species is likely to lead to errors of both type I and II in the results (Harvey & Pagel 1991). In the last decade, one of the most widely used approaches to correct for such 'phylogenetic effects' was the 'independent contrasts method' (Felsenstein 1985). The principle of this method is to assess the degree of differentiation between two species and then comparing these contrasts. In this study, values of the brain mass of the ancestral species would be the average brain

mass of its descendant species. Therefore the more resolved the phylogeny, the greater the power of the analysis due to a higher number of contrasts (Felsenstein 1985; Harvey & Pagel 1991). This method commonly assumes that traits (e.g. brain mass) evolved in the evolutionary history as Brownian motion (the degree of change in a trait between two species is proportional to the distance between these two species on the phylogenetic tree) and changes in the investigated trait that occur on different branches are independent (Felsenstein 1985).

A more recent and versatile approach that has been proposed is the phylogenetic general linear model (PGLM), procedure described by Gage & Freckleton (2003), using a variance-covariance matrix extracted using APE (Paradis *et al.* 2004). This procedure will be used in the present study to control for non-independence between the species due to shared ancestry (Harvey & Pagel 1991). The main principle of this method is the employment of maximum likelihood (ML) in order to estimate an index of phylogenetic dependence, λ, which assesses the degree to which shared ancestry explains the data (Pagel 1999; Freckleton *et al.* 2002) When $\lambda = 0$, the 'phylogenetic signal' to the data is null and alternative approach without control for phylogeny can be used whereas when $\lambda = 1$, independent contrast method could alternatively be used since the data fit a Brownian motion model. When $0 < \lambda < 1$, this intermediate value can be incorporated into the analysis to control for the specific degree of phylogenetic signal exhibited by the data to be analysed (Gage & Freckleton 2003).

In the present study, I used information from published supertrees to construct phylogenies for bats (Jones *et al.* 2002), primates (Purvis 1995), carnivores (Bininda-Emonds *et al.* 1999), insectivores (Grenyer & Purvis, 2003) and rodents (Bininda-Emonds *et al.* 2007). For ungulates, I used the tree compiled by Shultz & Dunbar (2006) based on three different sources (Gatesy *et al.* 1997; Flagstad *et al.* 2001; Murphy *et al.* 2001). Branch lengths were largely unknown, so unit branch lengths were assumed throughout (Freckleton *et al.* 2002). In all cases, brain mass and testis mass were log-transformed prior to analysis, and log-transformed body mass was included in all analyses as a covariate (Harvey & Krebs 1990).

I constructed a series of models to investigate potential effects of testis mass and sperm competition level on brain size evolution. In a general model including species from the full range of taxa investigated (bats, rodents, ungulates, primates, carnivores and insectivores), I looked for an influence of sperm competition (based on relative testis mass or sperm

competition level) on total brain mass. I then conducted the same test for each taxon separately, except for insectivores due to the small number of species ($n = 7$) available for this group in the dataset. Finally, because of differences between groups reported by Pitnick *et al.* (2006), these data were also re-analysed to test the expensive hypothesis separately in echolocating (formerly Microchiroptera) and non-echolocating (formerly Megachiroptera) bats.

In each analysis, brain mass was entered into the PGLM as the dependent variable, with body mass plus testis mass or mating system as the independent variables. I confirmed that testis mass is related to sperm competition level as expected (see **Section 6.2**; **Chapter 1**), by conducting further tests with testis mass as the dependent variable and body mass and sperm competition level as the independent variables (all data were log-transformed). I also conducted tests with only body mass as an independent variable to compare the Akaike's Information Criterion (AIC) of these models with the AIC of the model described above. If an investigated variable (testis mass or mating system) has a significant effect on brain size evolution, I expect the AIC of these models to be smaller that the AIC of the model with only body mass as the independent variable. Again, these analyses were conducted both within each mammalian group for which sufficient data were available and across the dataset as a whole.

6.4 Results

No significant negative relationship was found between testis mass and total brain mass, either across all species in the dataset, or within each of the mammalian orders tested separately (**Table 6.1a**). Indeed, in rodents, I found a marginally non-significant positive relationship between testis mass and brain mass ($n = 89$, $t = 1.86$, $P = 0.07$). Overall then, my analysis provides no evidence for the evolutionary trade-off between testis size and brain size predicted by the expensive sexual tissue hypothesis.

Next, I looked for evidence that brain size differs between species according to whether they typically experience 'high' or 'low' levels of sperm competition, where sperm competition levels were assessed on the basis of mating system classifications rather than testis size *per se* (see Methods). Here, as expected, species classed as having a 'high' sperm competition level

had significantly larger relative testis mass compared to those classed as having a 'low' sperm competition level, both across the dataset as a whole (n = 141, t = 6.17, $P < 0.0001$) and within 4 of the 5 taxonomic groups investigated separately (bats: n = 28, t = 5.45, $P < 0.0001$; rodents: n = 21, t = 3.69, $P = < 0.01$; ungulates: n = 28, t = 2.47, P = 0.02; primates: n = 41, t = 0.27, $P < 0.01$; carnivores: n = 23, t = 1.67, P = 0.11; for a possible explanation of the carnivore result, see Iossa *et al.* (2008)). However, consistent with the results for testis mass, I found no significant difference in total brain mass between species with contrasting levels of sperm competition based on mating system classifications, either across all the mammalian species in the dataset or within each of the groups analysed separately (**Table 6.1b**). Bats were the only exception to this pattern; as previously reported by Pitnick *et al.* (2006), for this group I found that high levels of sperm competition are associated with smaller brains (**Table 6.1b**).

Further analyses for bats reveal that the relationship between brain and testis mass is significant and negative for echolocating species (**Table 6.1a**), and 'low' sperm competition species in this group have larger brains than 'high' sperm competition species (**Table 6.1b**). For each model where the relationship between brain mass and testis mass or mating system was significant (**Table 6.1a** and **Table 6.1b**), the AIC increased when the tested variable was removed from the model (not shown) which confirms the importance of testis mass or mating system to explain variation in brain size (As expected, the AIC decreased when I removed the tested variable in the models where they do not have a significant effect). For the 26 echolocating species for which data on both testis mass and mating system are available, both trends are still apparent when the two explanatory variables are analysed separately (testis: n = 26, t = -2.00, P = 0.06; sperm competition level: n = 26, t = -2.82, $P < 0.01$). However, when both are combined in the same model, only sperm competition level (based on mating system classifications) approaches significance (**Table 6.2**). These patterns are strengthened if the alternative body mass measure (based on testis mass data sources – see **Section 6.3**) is used, resulting in a significant effect of sperm competition level (P = 0.03, not shown). The non-echolocating species also differ in brain size according to mating system classification (**Table 6.1b**), but in contrast to echolocating species there is a significant positive relationship between brain and testis mass.

Table 6.1 Phylogenetic general linear model analysis of total brain mass in relation to (a) testis mass and (b) sperm competition level classification (SCL), a dichotomous variable based on the degree of female promiscuity. The group "mammals" is composed of bats, rodents, ungulates, primates, carnivores and insectivores. In each model, body mass is included as a covariate.

	(a) Testes mass						(b) Sperm competition level (SCL)					
Taxon	*n*	ML λ	Variables	Estimate ± SE	t-Value	*p*	*n*	ML λ	Variables	Estimate ± SE	t-Value	*p*
Mammals	277	0.999	Body mass	0.50 ± 0.02	24.22	< 0.001	165	0.999	Body mass	0.56 ± 0.02	43.22	< 0.001
			Testis mass	0.03 ± 0.02	1.32	0.186			SCL	-0.02 ± .06	-0.35	0.72
Bats[1]	75	0.997	Body mass	0.70 ± 0.03	22.12	< 0.001	38	0.906	Body mass	0.73 ± .03	20.91	< 0.001
			Testis mass	-0.04 ± 0.02	-1.60	0.112			SCL	-0.19 ± 0.07	-2.39	0.02
Echolocating bats	61	0.896	Body mass	0.74 ± 0.04	19.76	< 0.001	32	< 0.001	Body mass	0.77 ± 0.04	19.87	< 0.001
			Testis mass	-0.07 ± 0.03	-2.69	0.009			SCL	-0.31 ± 0.08	-3.90	< 0.001
Non-echolocating bats	14	< 0.001	Body mass	0.61 ± 0.03	18.78	< 0.001	6	< 0.001	Body mass	0.69 ± 0.02	27.11	< 0.001
			Testis mass	0.09 ± 0.03	2.8	0.017			SCL	-0.30 ± 0.08	-3.72	0.03
Rodents	89	0.937	Body mass	0.38 ± 0.02	14.19	< 0.001	21	0.999	Body mass	0.42 ± .04	10.36	< 0.001
			Testis mass	0.05 ± 0.03	1.86	0.07			SCL	0.19 ± .12	1.60	0.13
Ungulates	28	0.999	Body mass	0.52 ± 0.06	7.64	< 0.001	28	0.999	Body mass	0.45 ± 0.05	8.26	< 0.001
			Testis mass	-0.06 ± 0.07	-0.71	0.48			SCL	0.14 ± 0.12	1.19	0.24
Primates	43	0.739	Body mass	0.63 ± 0.05	11.71	< 0.001	41	0.800	Body mass	0.60 ± .049	12.08	< 0.001
			Testis mass	-0.001 ± 0.004	-0.26	0.80			SCL	-0.27 ± 0.20	-1.36	0.18
Carnivores	35	0.999	Body mass	0.56 ± 0.06	9.84	< 0.001	36	0.651	Body mass	0.58 ± 0.03	16.87	< 0.001
			Testis mass	0.05 ± 0.08	0.63	0.53			SCL	0.02 ± 0.13	0.18	0.86

[1] Note that the results for bats differ slightly from Pitnick *et al.* (2006). The relationship between testis mass and brain mass was negative for bats, but did not reach statistical significance in my analysis, either in the model presented or with λ set to 0 or 1, or with branch lengths set according to Jones *et al.* (2005). The most likely explanation for this difference is the way in which the two different methods handle polytomies. In particular, owing to a poorly resolved phylogeny, the family Vespertillionidae contributes 5 independent contrasts to the analysis of Pitnick *et al.* (2006), despite the fact that 18 species from this family are included in the analysis; there is no obvious correlation between brain mass and testis mass in Vespertillionidae, a result which has a proportionately greater influence on my overall result.

Table 6.2 Phylogenetic general linear model analysis for echolocating bats of total brain mass in relation to body mass, testis mass and sperm competition level classification (SCL), a dichotomous variable based on the degree of female promiscuity. Compared to results described in **Table 6.1**, body mass, testis mass and SCL are here tested simultaneously in the same model.

n	ML λ	Variables	Estimate ± SE	t-Value	*p*
26	< 0.001	Body Mass	0.845 ± 0.065	12.94	< 0.001
		Testis Mass	-0.021 ± 0.06	-0.35	0.729
		SCL	-0.240 ± 0.131	-1.834	0.080

6.5 Discussion

No significant negative relationship was found between testis and brain mass across mammals in our dataset, or within any mammalian order investigated separately (bats, rodents, ungulates, primates, carnivores). Hence, I find no general evidence in support of the expensive sexual tissue hypothesis as applied to mammalian taxa. As similarly argued by Hladik *et al.* (1999) in relation to the expensive tissue hypothesis of Aiello & Wheeler (1995), it is not immediately obvious why the energetic costs of any two specific costly organs should impact directly on one another, instead of producing more diffuse effects on investment in a range of other costly organs or activities (Barton 2006). Aiello & Wheeler's (1995) expensive tissue hypothesis has also received mixed support beyond their original analyses for anthropoid primates. For example, Jones & MacLarnon (2004) reported evidence of a positive relationship between relative brain size and intestine length in bats, rather than the negative evolutionary relationship predicted, and Isler & van Schaik (2006) found no compelling evidence that the size of other energetically expensive organs (e.g. heart, lungs, gut) is systematically traded off against brain size in birds. Moreover, the mammalian brain is a highly complex organ with a large number of components and neuronal connections involved in many functions (Laughlin *et al.* 1998); thus, a simple energetic trade-off between any organ and overall brain mass may be unlikely (Hladik & Pasquet 2003).

Consistent with the results for relative testis mass, I find no evidence that total brain mass differs according to level of sperm competition based on our mating system classification across all mammals tested, or within each group tested separately except for bats (see below). Similarly, Schillaci (2006) found no interspecific differences in brain size of primates according to levels of female promiscuity. Here it is important to note that my 'level of sperm competition' classification, like the mating system classification of Schillaci (2006), is based on levels of female promiscuity, whereas earlier studies have placed greater emphasis on aspects of social organisation. In primates for example, bigger brains are found in harem and multi-male species compared to solitary and pair-bonded species (Shultz & Dunbar 2007), whereas sperm competition is high only in multi-male species (Harcourt *et al.* 1995). Consequently, primate brain size appears to correlate more closely with sociality than with sperm competition (see also Shultz & Dunbar 2007).

The only mammalian group for which I find evidence of a negative relationship between testis mass and brain mass was the echolocating bats (see also Pitnick *et al.* 2006). Might the expensive sexual tissue hypothesis then be applied as a special case to this group? Echolocating bats (which are typically insectivorous) are smaller compared to non-echolocating bats (which feed primarily on fruit) and so small body size could be one source of energetic constraint. This seems unlikely however, since the other group in my analysis with small body size, rodents, actually exhibited a positive trend in the relationship between testis mass and brain mass. Alternatively, the energetic costs of flight and echolocation are also substantial (Arita & Fenton 1997; Winter & von Helversen 1998) and so might act as a specific constraint in echolocating bats (Pitnick *et al.* 2006). However, although perhaps not directly comparable to bats (Winter & von Helversen 1998), evidence for birds tends not to support the idea that flight constrains investment in encephalization (Iwaniuk *et al.* 2004). Moreover, my results for echolocating bats suggest that mating system may be a better predictor of brain size than testis size *per se* (see also Shultz & Dunbar 2007). That is, when I tested simultaneously for an effect of both testis size and mating system classification on brain size in echolocating bats, only mating system approached significance. Hence on the basis of this analysis, I find limited support for the expensive sexual tissue hypothesis even in echolocating bats. Similarly, Dechmann & Safi (2009) reported that brain mass and testis mass in bats are no longer correlated after accounting for foraging strategy.

More broadly, there are a number of difficulties associated with applying the comparative method to understand patterns of brain size evolution, particularly in relation to hypotheses about sexual selection (Healy & Rowe 2007). Importantly, specific parts of the brain may vary independently of whole brain size (the 'mosaic brain evolution' hypothesis; Barton & Harvey 2000), and studies which seek to explain variation in the size of specific parts of the brain may often be more informative than those which focus on total brain size, particularly if such parts can be assigned a reasonably discrete function (Krebs 1990; Barton 1998; Barton 2004; Healy & Rowe 2007). Secondly, comparative analyses of brain components measured separately in males and females are also relevant to understanding potential effects of sexual selection on encephalization (Lindenfors *et al.* 2007), since sex differences in brain structure have already been established (Jacobs *et al.* 1990, Gahr *et al.* 2008). In zebra finches and canaries for example, the high vocal center and the robust nucleus of the archistriatum, which

are two subregions of the brain involved in singing, are larger in males than in females (MacDougall-Shackleton & Gall 1999). Therefore it seems likely that natural or sexual selection may influence male and female brains differently (Garamszegi *et al.* 2005a), and if sexual selection favours a larger overall brain size in males than in females, the effect of sperm competition on encephalization might be underestimated when using an average measure. Hence, to be tested rigorously, hypotheses involving a putative role of sexual selection in brain size evolution should be tested with sex-specific data on brains or brain components.

6.6 Conclusion

I conclude that there is no general trade-off between testis mass and brain mass in mammals, and consequently that there is no evidence that the expensive sexual tissue hypothesis can provide a general explanation for the evolution of mammalian brain size. Moreover, as noted by Dunbar & Schultz (2007), energetic or developmental explanations are mainly aimed at identifying constraints on brain size evolution, rather than the selection pressures through which individuals might benefit from larger brains. Although the energetic costs of large brains must indeed be somehow accommodated, there is perhaps no reason to assume that this is achieved through a simple and invariant mechanism, such as a trade-off against the size of another organ. Instead, there may be a variety of complex and varying trade-offs among body size, life histories, energy acquisition rates, and energy allocation to a range of organs of which the brain is just one (Isler and & Schaik 2006; Barrickman *et al.* 2008). I suggest, therefore, that understanding the costs of brain size evolution requires a broader approach to these trade-offs than has commonly been used in the past.

Chapter 7: General conclusion

7.1 Chapter overview

The first part of this chapter will summarize the principal results presented in this thesis (**Section 7.2**). Some of these findings will then be discussed in relation to current and central questions in evolutionary and behavioural ecology such as complex relationships between pre- and post-copulatory sexual selection (**Section 7.3**) and benefits of polyandry (**Section 7.4**). New opportunities for the study of sexual selection and sperm competition in bank voles, including the role of androgens and plasticity in social status, will then be proposed (**Section 7.5**). Finally, I will conclude this chapter and this thesis by highlighting the importance of sperm competition in current studies in evolutionary ecology and by suggesting possible applications of my findings in conservation biology (**Section 7.6**).

7.2 Summary of results

In mammals, females generally prefer dominant males over subordinates (Luque-Larrena *et al.* 2003; Havlicek *et al.* 2005) and studies in bank voles have shown that this species follows the rule (Horne & Ylönen 1996; Kruczek 1997). Females might choose dominant males on the basis of the higher concentration of protein found in their urine (**Chapter 4**). Although male mate choice is generally less studied than female mate choice (Dewsbury 2005), males can also express a pre-copulatory preference toward specific females. Results discussed in **Chapter 2** reveal a male preference for unrelated females in bank voles. This behaviour is likely to promote inbreeding avoidance, independently of male social status. Since mating with related females is costly in terms of reproductive success (Kruczek 2007), males might maximize their fitness by being choosy. However, contrary to theoretical predictions (Kokko & Johnstone 2002), after mating to satiety, males do not show a preference for unrelated females. These results imply than potential benefits of mate choice may vary among males according to recent mating activity but not social dominance. Male bank voles in this study

showed no preference with regard to female body mass or age. Further experiments on male mate preference could also take into account the females parasite infection status. Indeed in natural populations, individual survival is dependent on the presence of parasites (Kallio *et al.* 2007) and male bank voles could exhibit a preference for healthy females in order to maximize their fitness (see also Dunn *et al.* 2006).

In post-copulatory competition, dominant male bank voles have higher reproductive success than subordinate males and as suggested by Klemme *et al.* (2006a), this higher fitness can come from an advantage in sperm competition, female cryptic choice or both. Dominant males perform more intromissions prior to the first ejaculation than do subordinate males (**Chapter 4; Table 7.1**), which might decrease the female propensity to remate (Huck & Lisk 1986). However, this result combined with the wider (but not longer) baculum found for dominant males (**Chapter 3; Table 7.1**) suggests that these high quality males provide more stimulation to females during copulation. Consequently, females might select sperm from these males if their sons will inherit this ability to stimulate females intensively (Fisherian model of sexual selection) and if this higher stimulation is an honest indicator of intrinsic quality (good-genes hypothesis) (see Kokko *et al.* 2002 for a recent review of the different models of sexual selection). The higher reproductive success of dominant males might therefore be explained by an advantage in female cryptic choice. In red jungle fowl, females preferentially select sperm from unrelated males but to counteract this female cryptic choice, males invest more sperm when mating with related females (Pizzari *et al.* 2004). Similarly in bank voles, subordinate males should invest more sperm than dominant males during a mating. This is also predicted by theoretical models designed to understand optimal ejaculate allocation strategies that males should adopt in sperm competition according to their social status (Parker *et al.* 1990a). However, results from **Chapter 4** show that dominant males invest more sperm per ejaculate than subordinates (**Table 7.1**). This difference in sperm allocation strategy concurs with the larger testes and epididymis found for dominant males (**Chapter 4; Table 7.1**). Nonetheless, the quality of the ejaculate (i.e. sperm motility), another determinant of the male fertilization efficiency (Snook 2005), and the total number of ejaculations performed by males during a mating bout do not differ between dominant and subordinate males (**Chapter 4**). Dominant males also have heavier seminal vesicles (**Table 7.1**) suggesting that these males can invest more in seminal fluid proteins even if no difference in copulatory plug production was found according to male social status. Overall, contrary to theoretical predictions (Parker 1990a; Tazzyman *et al.* 2009), dominant male bank

voles invest more in ejaculate expenditure than subordinate males, which is likely to explain their higher reproductive success (Klemme *et al.* 2006a). However, it is also possible that the advantage of dominant males in terms of sperm competition is explained to some extent by female cryptic choice since they also provide more stimulation to females during copulation.

Results from **Chapter 5** reveal that male bank voles respond to the average level of sperm competition by investing more in seminal vesicles. Indeed, males who experienced a high level of competition in their environment have heavier seminal vesicles (but not testes) compared to males who experienced a low level of competition. Therefore, it is likely that the seminal fluid proteins produced by this accessory gland play a role in sperm competition, as it is the case in some species of insects (e.g. Crudgington *et al.* 2009; Wigby *et al.* 2009). In rodents the size of seminal vesicles is positively correlated with the level of sperm competition (Ramm *et al.* 2005) and the divergence rate of a seminal fluid protein (SVS II) involved in copulatory plug production correlates with the level of sperm competition (Ramm *et al.* 2009). Subsequent experiments should focus on strategic allocation of the seminal fluid proteins and copulatory plug in relation with the risk or intensity of sperm competition. Other potential functions of seminal fluid proteins such as an inhibition of female remating or an enhancement of sperm motility (Poiani 2006) also deserve more attention in sperm competition studies in mammals.

It is important to acknowledge that intra-specific variations in male androgen levels might explain results from the **Chapter 3**, **4** and **5**. Indeed, androgens such as testosterone influence various aspects of male reproduction such as sexual behaviour or spermatogenesis (Wingfield *et al.* 1990; Malo *et al.* 2009) and therefore mating success (Andersson 1994). For instance in grey-headed flying-foxes (*Pteropus poliocephalus*), males that control the largest harem also have the higher level of circulating plasma testosterone (Klose *et al.* 2009) probably because they are more aggressive (Muller & Wrangham 2004; Klose *et al.* 2009). More generally levels of testosterone are positively related with social rank (Rudolfsen *et al.* 2006; Setchell *et al.* 2008; but see Sapolski 1991).

In this study, I found that dominant males have bigger testes. There is now evidence at the inter- and intra-specific level that there is a positive correlation between testes size and the level of circulating T (Garamszegi *et al.* 2005b; Denk & Kempanaers 2006; Parapanov *et al.* 2009). Therefore, we can expect that dominant males in my study have a higher level of

testosterone (see also Mills *et al.* 2009a). This could explain why dominant males have larger preputial glands (Jean-Faucher *et al.* 1985), larger seminal vesicles (Kashiwagi *et al.* 2005) and larger baculum (Dixson & Anderson 2004). Since androgens decrease immune responses (Folstad & Karter 1992), antibodies are less likely to bind sperm cells and the quality of the ejaculate should therefore be increased (Skau & Folstad 2003, 2005) which is not the case in our study (but see Kruczek & Zatorska 2008). However, it is important to outline that our measurements of sperm motility were made in the absence of seminal fluid proteins, which might determine sperm motility within the female reproductive tract (Poiani 2006).

Further experiments should take in account the role of testosterone in the regulation of the male sexual behaviour and physiology. High circulating levels of testosterone can be costly for individuals since it decreases immune function (Folstad & Karter 1992) and increases risk of mortality (Marler & Moore 1998). Therefore it would be interesting to compare the mortality risk of dominant and subordinate male bank voles and their long-term reproductive success. Moreover, information on the level of testosterone but also glucortocoids should be obtained before and after the pairing in order to understand if subordinate male bank voles have a natural low level of these hormones or if this level is decreased by the presence of a competitor in the social environment (see also Rogers & Hendrie 1983; Sapolski 1991).

			Dominant > Subordinate	Subordinate > Dominant	Dominant = Subordinate	Chapter
Pre-copulatory competition		Body mass			x	4
		Body length			x	4
		Preputial gland mass	x			4
		Protein concentration in urine	x			4
		Protein:creatinine concentration in urine			x	4
Post-copulatory competition		Testis mass	x			4
		Epididymis mass	x			4
		Seminal vesicle mass	x			4
		Number of sperm (cauda epididymis)			x	4
		Number of sperm in the first ejaculate	x			4
		Sperm motility			x	4
		Copulatory plug mass			x	4
	Genitalia					
		Baculum total length			x	3
		Baculum central length			x	3
		Baculum central width			x	3
		Baculum basal width	x			3
		Penile spines length			x	3
		Penile surface covered by spines			x	3
		Penile spines density			x	3
	Copulatory behaviour					
		Number of intromissions	x			4
		Number of mounts			x	4
		Number of ejaculations			x	4
		Intromission latency			x	4
		Ejaculation latency			x	4
Litter size					x	4

Figure 7.1 Comparisons between dominant and subordinate male bank voles of different traits investigated in Chapters 3 and 4. Dominant > Subordinate means that this trait was significantly higher for dominant males; Subordinate > Dominant means that this trait was significantly higher for subordinate males and Dominant = Subordinate means that there was no significant difference on the trait between dominant and subordinate males.

7.3 Pre- and Post-copulatory sexual selection

Assumptions made in sperm competition studies generally encapsulate the evolutionary trade-off between investment in structures playing a role in mate location, acquisition or fidelity (pre-copulatory competition) and structures related to ejaculate expenditure (post-copulatory competition). In others words, sexual competition in some species will be mediated predominantly through pre-copulatory means and in other species through post-copulatory means, according to the particular reproductive ecology and evolutionary history of the species in question. Selection will favor differential investment in traits affecting male reproductive success leading to the greatest net gains in fitness. Some recent evolutionary biology studies have investigated these trade-offs at the inter-specific level through the use of comparative studies. Although an evolutionary trade-off between plasticity in horn growth (structure involved in pre-copulatory competition) and plasticity in testes growth (structure involved in post-copulatory competition) has been reported in *Onthophagus* species (dung beetles) (Simmons & Emlen 2006), comparable evidence is lacking in vertebrate taxa. For instance, results from the test of the 'expensive sexual tissue hypothesis' (Pitnick *et al.* 2006) suggest that in several mammalian orders, brain mass, used as a proxy of pre-copulatory investment[1] does not trade-off with testes mass, except perhaps in echolocating bats (but see **Chapter 6**).

At the intra-specific level, the relation between pre- and post-copulatory sexual selection can take several forms depending on the species studied. For example, in the arctic charr (*Salvelinus alpinus*), subordinate males are disadvantaged in pre-copulatory competition and invest more in ejaculate expenditure than dominant males (Rudolfsen *et al.* 2006; see also Froman *et al.* 2002; Neff *et al.* 2003; Simmons & Emlen 2006). However, as pointed out in the previous section of this chapter, dominant male bank voles are better competitors in both pre- and post-copulatory competition. This positive association between investments in these two components of sexual competition seems more common in mammals (Koyama & Kamimura 1999; Faulkes & Bennett 2001; Malo *et al.* 2005b; but see Hosken *et al.* 2008; Thomas & Simmons 2009) but in several of these species the role of social constraints needs to be taken into account. For example in naked mole-rats, dominant males can suppress the reproductive activity of non breeding individuals who will have more abnormal sperm in their

[1] In mammals, it has been suggested that individuals with high cognitive capacities (i.e. larger relative brain mass) can develop better strategies to improve mate choice and avoid cuckoldry (Shultz & Dunbar 2007).

reproductive tract compared to dominant males (Faulkes & Bennett 2001; see also Fitzpatrick *et al.* 2006). Therefore in such species, the low quality ejaculate found in subordinate males might be a result of social constraints rather than adaptive strategies (Pizzari *et al.* 2007). In bank voles, although the possible suppression of subordinate reproduction elicited by dominant males cannot be completely ruled out, it might be possible as suggested by Mills *et al.* (2009b) that two different reproductive tactics, perhaps genetically determined, coexist (see **Section 7.4** and **Section 7.5**).

7.4 Why do female bank voles mate multiply?

Results from this thesis highlight a paradox already acknowledged by Klemme *et al.* (2007a). That is, if female bank voles show a preference for dominant males in pre- and maybe post-copulatory competition (Kruczek 1997; **Chapter 3**), and if dominant males provide more sperm than required to obtain a large litter size (**Chapter 4;** Klemme *et al.* 2007a), why do females still solicit copulations with subordinate males?

In bank voles, mating with dominant males potentially provides direct and indirect benefits to females. It can directly improve their fitness via a higher number of offspring (Klemme *et al.* 2006a) and enhance the rate at which their offspring achieve sexual maturity (Kruczek & Zatorska 2008). Male dominance and quality are also heritable in this species (Horne & Ylönen 1998; Oksanen *et al.* 1999; but see also Wilson & Nussey 2010). Since female bank voles do not improve their reproductive success through an adjustment of the maternal effort (Oksanen *et al.* 1999), mate choice based on male social rank would seem adaptive and there is evidence that, in a simultaneous choice situation, females spend more time investigating dominant than subordinate males (Horne & Ylönen 1996; Kruczek 1997).

Recently, Klemme *et al.* (2008) pointed out the first evidence for benefits of polyandry in bank voles. They found that offspring of polyandrous females, especially sons, have a higher reproductive success compared to offspring of monandrous females. Even if the causal mechanism explaining this result is still unclear (Klemme *et al.* 2008), it might be possible that females promote polyandry to reduce genetic incompatibilities (Zeh & Zeh 1996; Zeh & Zeh 1997; Tregenza & Wedell 2002; Firman & Simmons 2008b). Under this scenario, even if dominant males are able to skew paternity in their favour by investing more sperm than subordinates, females might still be able to select sperm (Eberhard 1996) from the most

genetically compatible males (see also Pizzari *et al.* 2004). Therefore, polyandry in this species might be favoured since its benefits exceed those of mating with dominant males only. Furthermore, female polyandry, including mating with dominant and subordinate males, might decrease the risk of infanticide (Klemme & Ylönen 2010; see also Jennions & Petrie 2000) or prevent fitness decline in case of a deterioration of the environment, since the competitive abilities of dominant males' sons can be environment-dependent (Mills *et al.* 2007). Dominant males are also likely to have a higher mating rate than subordinates and are therefore more likely to suffer from sperm depletion (Preston *et al.* 2001) or from a decrease in sperm quality (Cornwallis & Birkhead 2007). Therefore, female bank voles might decrease the risk of sperm limitation due to exclusive mating with dominant males by soliciting mating with subordinate males. If females benefit from mating with males of different social status, this could also explain why dominant-subordinate relationships persist in bank vole populations despite heritability of male dominance and quality (Horne & Ylönen 1998; Oksanen *et al.* 1999; and see the following section), and despite higher investment in sperm and copulation by dominant males.

7.5 Perspectives for further studies

Recently, Mills *et al.* (2009b) found a trade-off between investment in reproductive effort (measured as mating success in a competitive situation) and survival in bank voles. The males who invest more in reproductive effort than in survival, and who are likely to be the dominants in our study (even if the method I used to assess male quality differs from the one used in Mills *et al.* 2007; Mills *et al.* 2009a; Mills *et al.* 2009b), have the same fitness as males who invest more in survival and are likely to be the subordinates in our study. Future experiments should measure the survival rate of pairs of dominant-subordinate males since it could explain why some males invest in both pre- and post-copulatory competition and some males do not, in order maybe to increase their lifespan. What's more, males who invest more in reproductive effort have a higher level of testosterone (Mills *et al.* 2009a). An elevated level of testosterone generally entails a decrease of the immune response (Hau 2007; Mills *et al.* 2009a) that in return increases the quality of the ejaculate (Skau & Folstad 2004). Subsequent works performed on differences in male reproductive strategies in bank voles and more generally in mammals should measure the level of androgen hormones (e.g. testosterone; 11-ketotestosterone) that are likely to interact with male behaviour and ejaculate

quality. Moreover, the use of a standardized method to establish dominant-subordinate relationships in bank voles would help to compare results between studies.

More attention should also be paid in the future to the selective mechanisms maintaining variation in reproductive strategies between males since, as emphasized by Cornwallis and Birkhead (2007), between-subject designs do not allow us to disentangle between phenotypic plasticity and genetic polymorphism of sexual traits. In fowl, *Gallus gallus*, dominant and subordinate males differ in behaviour (Cornwallis & Birkhead 2008) and sperm allocation strategy (Cornwallis & Birkhead 2006). However, these phenotypes appear to be plastic since they change in response to the social environment (i.e. when the social status of these males is reversed) (Cornwallis & Birkhead 2007, 2008; Pizzari *et al.* 2007). To date, attempts to reverse the social status of bank voles have been unsuccessful since dominant males paired together generally maintain a high investment in scent marking (Lemaître, Pers. obs.). Moreover, it is unclear which fitness gains could make adopting a subordinate position an adaptive tactic for male bank voles.

7.6 Concluding remarks

To celebrate Darwin's birthday and legacy, the 2009 summer conference of the Association of the Study of Animal Behaviour (ASAB) was focused on sexual selection, nearly 150 years after the publication of '*The Descent of Man, and Selection in Relation to Sex*'. During this conference, a third of the presentations concerned sperm (or testis), emphasizing the central place of sperm competition in current studies on sexual selection. This thesis modestly supports the importance of sperm competition in sexual selection in bank voles but suggests that broader knowledge of the different traits involved in post-copulatory competition (e.g. seminal fluid proteins, testosterone) is now required to understand more completely the evolution of male and female physiology, behaviour and alternative mating strategies.

Also, if the pattern found here, namely dominant males producing larger ejaculates (**Chapter 4**) or better quality ejaculates (e.g. less abnormal sperm; Kruczek & Styrna, 2009), is widespread in mammals, this could be a great help in programs of assisted reproduction. For instance females insemination with sperm from dominant males might improve the conservation of endangered species (review in Roldan & Gomendio 2009), particularly if dominance and sperm quality are heritable (Horne & Ylönen 1998; Oksanen *et al.* 1999; Mills

et al. 2007). Moreover, if the quantity of seminal fluid protein is a determinant of mating rate, artificial selection of males with larger seminal vesicles (**Chapter 5**) could also be used more widely in animal breeding programs to increase the number of inseminations.

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Appendix: Comparative data

Testes mass, body mass, brain mass and sperm competition level for rodents, primates, ungulates, carnivores and insectivores*. Testes mass (TM) is the average combined mass of both (right and left) testes, measured from multiple adult males in reproductive condition; brain mass (BrM) is total brain mass, measured as an average from different adult individuals; body mass (BM) data were obtained from the same source as brain mass data where possible; sperm competition level (SCL) is a dichotomous classification based on mating system. Species classified as having relatively high sperm competition include those with polyandrous, polygynandrous, and promiscuous mating systems, and those classified as having relatively low sperm competition include species with polygynous and monogamous mating systems. When data were available from multiple sources in the literature for testes mass, body mass and brain mass, I used the mean of these data weighted by the sample size provided by each study. All brain and body mass data should be considered as an average size for males and females.

	Species Name	TM (g)	BM (g)	BrM (g)	SCL	Sources
Rodents	*Aethomys chrysophilus*	0.862	117	1.84		1; 2
	Aethomys hindei	2.768	146.3	2.01		1; 2
	Aethomys namaquensis	1.476	79.4	1.48		1; 2
	Ammospermophilus leucurus	2.14	106	2.92	High	1; 3; 4
	Apodemus flavicollis	0.891	32.3	1.29		1; 2
	Apodemus sylvaticus	0.788	18.6	1.17		1; 2
	Arvicola terrestris	0.78	144.15	1.865		1; 3; 4
	Cavia aperea	0.95	524.9	5.86	Low	1; 5
	Cavia porcellus	4.1	971	4.3		3; 4; 6
	Chaetodipus formosus	0.21	20.9	1.13		1; 4
	Chinchilla lanigera	5.2	432	5.5		3; 4
	Chiropodomys gliroides	0.38	25.3	1.29		1; 2
	Clethrionomys glareolus	0.646	17.9	1.11	High	1; 3; 4
	Cynomys leucurus	4.88	992.1	6.28		1; 8
	Cynomys ludovicianus	0.928	793.75	6.6		1; 3; 9
	Dasyprocta leporina	5.03	2800	20.3		3; 10
	Dicrostonyx groenlandicus	0.092	68.4	1.31		1; 11
	Dipodomys merriami	0.45	37.9	1.64		1; 3; 4; 12
	Dipodomys microps	0.4	60	1.8		1; 3; 4; 12
	Dipodomys ordii	0.53	54	1.97		1; 4; 12
	Dipodomys panamintinus	0.52	74	1.54		4; 12
	Galea musteloides	2.77	375	3.31	High	1; 5
	Gerbillus pyramidum	0.81	40.9	1.47		1; 13
	Jaculus jaculus	0.58	55.2	1.8		1; 3; 14
	Lophuromys flavopunctatus	1.138	60	1.6		1; 2
	Marmota monax	7.4	4199	11.24	High	1; 3; 4
	Megadontomys thomasi	2	77	2.01		3; 15
	Melomys cervinipes	2.782	70	1.9		1; 2
	Melomys rubex	1.872	49.7	1.86		1; 2
	Melomys rufescens	1.342	54.7	1.88		1; 2
	Mesembriomys gouldii	5.498	1110	5.18		1; 2
	Mesocricetus auratus	3.17	13.3	1.12		1; 3; 4

Micromys minutus	0.15	6.4	0.88		1; 2
Microtus agrestis	0.804	22.3	1.16		1; 4
Microtus arvalis	0.424	30.4	1.14		1; 4
Microtus californicus	0.27	43.15	1.37	Low	3; 4
Microtus montanus	0.38	39.85	1.28	High	1; 3; 16
Microtus ochrogaster	0.52	43.9	1.3	Low	1; 3; 16
Microtus pennsylvanicus	0.58	37.15	1.36	High	1; 3; 16
Microtus pinetorum	0.07	24.9	1.15	Low	1; 16
Mus musculus	0.119	14	0.44	Low	2; 6; 17
Myocastor coypus	4.4	9070	17.68		1; 18
Neotoma micropus	1.206	378.3	3.25		1; 19
Notomys alexis	0.045	37	1.55		1; 2; 3; 4
Ondatra zibethicus	5.31	1236.5	5.76	Low	1; 3; 4
Perognathus longimembris	0.07	8.15	0.93		1; 3; 4; 12
Peromyscus boylii	0.3	16.3	1.37		1; 15
Peromyscus californicus	0.2	34.2	1.54		1; 15
Peromyscus crinitus	0.12	14.7	0.875		1; 3; 15
Peromyscus eremicus	0.232	20.65	0.885		1; 3; 15
Peromyscus gossypinus	0.68	26.6	1.42		1; 15
Peromyscus leucopus	0.25	21.8	1.24		1; 15
Peromyscus maniculatus	0.393	20.95	0.88	High	1; 3; 4
Peromyscus megalops	1.48	71	1.8		1; 3; 15
Peromyscus mexicanus	0.43	53.4	1.63		1; 15
Peromyscus polionotus	0.1	14	1.12	Low	1; 15; 20
Peromyscus truei	0.47	30	1.36		1; 3; 15
Podomys floridanus	0.58	27.3	1.54		1; 15
Praomys tullbergi	1.43	37.2	1.45		1; 21
Pseudomys australis	2	50	1.75		1; 2
Rattus exulans	1.893	42.1	1.69		1; 22
Rattus fuscipes	4.262	135.7	2.22		1; 2; 3
Rattus lutreolus	4.434	92.85	2.23		1; 2; 3
Rattus norvegicus	4	269	2.09		4; 7
Rattus rattus	3.467	150	1.92		2; 3

	Rattus sordidus	3.522	158	2.29		2; 3
	Rattus tunneyi	4.865	243	2		2; 3
	Rhabdomys pumilio	0.909	55	1.28		2; 3
	Sciurus aestuans	2.6	626.75	7.44		1; 3; 4
	Sciurus carolinensis	5.18	574.5	7.76	High	1; 4
	Sciurus niger	6.9	809.9	9.31		1; 4
	Sciurus vulgaris	2.81	331.8	6.34	High	1; 4
	Sigmodon hispidus	1.73	140.25	1.455		1; 3; 4
	Spermophilus beecheyi	9.05	587.25	5.71	High	1; 3; 4
	Spermophilus beldingi	2.5	263	3.87		3; 23
	Spermophilus lateralis	3.25	166	3.6	High	1; 3; 4
	Spermophilus townsendii	0.624	199	2.65		1; 24
	Spermophilus tridecemlineatus	1.27	175	3.02		3; 25; 26
	Tamias amoenus	0.62	50.8	1.98	High	1; 4
	Tamias minimus	0.72	45.3	2.19	High	1; 4
	Tamias palmeri	0.86	60.8	2.58		1; 4
	Tamias panamintinus	7.8	51.2	2.42		1; 4
	Tamias striatus	1.7	93.65	2.73		1; 3; 4
	Tamias townsendii	0.82	89.4	3.03	High	1; 4
	Tatera afra	7.74	65	2.15		1; 4
	Tatera brantsii	5.31	91.7	2.15		1; 4
	Tatera indica	6.41	139.85	2.43		1; 3; 4
	Uranomys ruddi	0.348	33.6	1.36		1; 2
Primates	*Aloutta palliata*	23	7260	55.1	High	28; 29
	Aotus trivergatus	1.2	1020	18.2	Low	28; 29
	Ateles geoffroyi	13.4	7940	110.9	High	27; 29
	Avahi laniger	2.29	1300	7.4	Low	2; 29; 38
	Callithrix jacchus	1.3	320	7.9	High	27; 29
	Cebuella pygmaea	0.33	130	4.15	High	27; 29
	Cebus apella	4.64	3000	10	High	27; 29
	Cercophitecus ascanius	3	5360	67	High	27; 29
	Cercopithecus aethiops	13	4950	59.8	High	28; 30

Cheirogaelus major	2.3	340	6.8		27; 29
Colobus polykomos guereza	10.7	10250	76.6	Low	28; 29
Erythrocebus patas	7.2	10000	108	High	27; 29
Galago senegalensis	1.66	220	4.8	High	3; 29; 38
Galagoides demidovii	0.85	70	3.4	High	27; 29
Gorilla gorilla	29.6	169000	505.9	Low	28; 29
Hylobates agilis	6.32	6000		Low	28
Hylobates lar	5.5	5500	107.7	Low	28; 29
Hylobates moloch	6.1	5440	113.7	Low	28; 29
Lagothrix lagothrica	11.2	5220	96.4	High	28; 29
Leontopithecus rosalia	1.48	550		Low	27
Loris tardigradus	1.8	270	6.8		27; 29
Macaca arctoides	48.15	10510	76.8	High	28; 29
Macaca fasciularis	35.2	4420	69.2	High	28; 29
Macaca mulatta	46.2	9200	95.1	High	28; 29
Macaca nemestrina	66.7	9980	106	High	28; 29
Macaca radiata	48.2	8650	104.1	High	28; 29
Microcebus murinus	2.49	70	1.78	High	27; 29
Miopithecus talapoin	5.2	1250		High	27
Nasalis larvatus	11.8	20640	94.2	Low	28; 29
Nycticebus coucang	1.2	1058	12.2	High	3; 29; 38
Pan troglodytes	118.8	44340	410.3	High	27; 29
Papio anubis	93.5	26400	93.5	High	28; 29
Papio cynocephalus	52	24320	169.1	Low	28; 29
Papio hamadryas	27.1	20170	142.5	Low	28; 29
Papio papio	88.9	31980	165.3	High	28; 29
Papio ursinus	72	31750	214.4	High	28; 29
Pongo pygmaeus	34.2	69000	413.3	Low	28; 29
Presbystis entellus	11.1	17000	135.2	Low	28; 29
Presbystis obscura	4.8	7450	67.6	Low	28; 29
Presbytis cristata	6.2	6580	64	Low	28; 29
Presbytis rubicunda	3.4	6230	92.7	Low	28; 29
Saguinus midas	1.83	570	10.3	High	27; 29

	Saguinus oedipus	3.4	520	9	High	28; 29
	Saimiri sciureus	3.2	780	24.4	High	28; 29
	Theropithecus gelada	17.1	20400	131.7	Low	28; 29
	Varecia variegatus	2.63	3471	30.8	Low	3; 29; 38
Ungulates	*Aepyceros melampus*	120	58500	179	High	31; 32
	Alces alces	106	200000	435	Low	31; 32
	Antilocarpa americana	76	24000	165	High	31; 32
	Capreolus capreolus	43	16850	100	Low	31; 32
	Cephalophus sylvicultor	33.25	45000	170	Low	31; 38
	Cervus elephus	218	90175	311	High	31; 32
	Dama dama	133	56234	215	High	31; 32
	Equus przewalskii	133	361450	586	High	31; 38
	Gazella dorcas	31.46	15000	66	Low	31; 38
	Giraffa camelopardalis	1074	581750	704	High	31; 32
	Hemitragus jemlahicus	26.7	70000	166	High	31; 32
	Hippopotamus amphibus	650	1955000	569	Low	31; 32
	Hylochoerus meinertzhageni	500	200000	140	High	31; 32
	Kobus leche	67.85	123000	223	High	31; 38
	Odocoielus virginianus	76	41960	144	Low	31; 32
	Oryx gazella	37.9	130000	274	Low	31; 38
	Phaecochoerus aethiopicus	93	68160	132	Low	31; 32
	Rangifer tarandus	132	95225	270	High	31; 32
	Sus scrofa	36	66900	180	Low	31; 32
	Sylviacapra grimmia	21.25	11900	76	Low	31; 38
Carnivores	*Acinonyx jubatus*		58560	111.05	High	33; 34
	Alopex lagopus	4.06	3190	35.52	Low	33; 35; 36
	Bassariscus astutus		840	16.44	High	33; 36
	Canis aureus	13.3	8760	72.24		33; 35
	Canis latrans	15.4	10490	88.23	Low	33; 35; 36
	Canis lupus	27.38	33110	131.63	Low	33; 35; 36
	Canis mesomelas		7690	56.83	Low	33; 37

Chrysocyon brachiurus	12.68	23100	120.3		33; 35
Crocuta crocuta	9.85	66020	144.03		33; 35
Cryptoprocta ferox	11.15	9490	32.14	High	33; 38
Cuon alpinus	6.35	17640	94.63	Low	33; 38
Cynictis penicillata	1.98	590	10.49		33; 35
Enhydra lutris		27940	125.21	High	33; 36
Felis chaus	4.62	7030	39.25		33; 35
Felis silvestris	1.38	4660	37.34	High	33; 35
Gulo gulo	17	11130	78.26	High	33; 35; 36
Herpailurus yagouaroundi		7460	40.04	High	33; 36
Leopardus pardalis		13460	63.43	High	33; 36
Lutra canadensis	18	7320	52.98	High	33; 35; 36
Lutra lutra	4.05	8670	42.1	Low	33; 38
Lynx lynx	7.2	11030	70.11		33; 35
Lynx rufus		6110	57.97	High	33; 36
Martes americana		860	15.8	High	33; 36
Martes pennanti	7.76	3460	31.82	High	33; 35; 36
Meles meles	14.4	13970	57.45	High	33; 35
Mellivora capensis	54	8080	72.97		33; 35
Mephitis mephitis	5.05	2360	10.28	Low	33; 35; 36
Mustela erminea	2.64	131.5	4.05	High	3; 33; 36
Mustela frenata	1.88	230	4.01	High	33; 35; 36
Mustela nigripes		580	8.5	High	33; 36
Mustela nivalis	0.43	70.5	1.83	High	3; 33; 36
Mustela putorius	3.14	1030	8.25		33; 35
Mustela vison	4.95	837.5	7.85	High	3; 33; 35
Nyctereutes procyonoides	9.18	7460	28.5		33; 35
Panthera leo	55	124235	219.815		3; 33; 35
Panthera onca	27.5	85630	151.41	High	33; 35; 38
Panthera tigris	24.4	184085	290.33	Low	3; 33; 38
Potos flavus		2050	28.365	High	3; 33; 39
Procyon lotor	13.72	6170	40.04		33; 36
Puma concolor		50400	125.21	High	33; 35

	Spilogale putorius	5.4	540	5	High	33; 35; 36
	Suricata suricatta	1.3	730	10.28		33; 35
	Taxidea taxus	35.56	4060	48.91	High	33; 35; 36
	Urocyon cinereoargentus	5.07	3630	40.85	Low	33; 35; 36
	Urocyon littoralis		2050	27.66	Low	33; 36
	Ursus americanus		109950	259.82	High	33; 36
	Ursus arctos	92	298870	336.97	High	33; 35; 36
	Vulpes velox		2200	32.14	Low	33; 36
	Vulpes vulpes	9	4760	45.46	Low	3; 4; 33
Insectivores	*Erinaceus europaeus*	2.31	812.5	3.635		1; 3; 4; 6
	Neomys fodiens	0.21	16.25	0.305		1; 3; 4
	Scalopus aquaticus	2.2	39.8	1.32		1; 3; 4
	Sorex araneus	0.28	7.5	0.25	High	3; 4; 40
	Sorex cinereus	0.1	3.9	0.17		1; 3; 4
	Sorex minutus	0.1	3.9	0.15		1; 4
	Sorex palustris	0.15	11.9	0.31		1; 4

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*Data for bats are not reproduced here and were taken from the single source:

Pitnick, S., Jones, K. E. & Wilkinson, G. S. 2006. Mating system and brain size in bats. *Proceedings of the Royal Society of London. Series B*, **273**: 719-724.